THE PROTECTIVE ROLE OF PINK1/PARKIN-MEDIATED MITOPHAGY IN SONODYNAMIC THERAPY

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The Protective Role of PINK1/Parkin-mediated Mitophagy in Sonodynamic Therapy

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Abstract

Over the past years, several major treatments have been developed during the study of cancer therapies, such as radiotherapy, chemotherapy, and surgery (Al-Sarraf, LeBlanc et al. 1998, Hüser, Michalski et al. 2008). Although these methods are significantly beneficial to patients, their drawbacks, such as therapeutic inefficiency and serious adverse effects, have prompted people to seek alternative treatments. Based on the synergistic effect of low-intensity ultrasound and sonosensitizer, sonodynamic therapy (SDT) is a promising non-invasive treatment of cancers, which has been demonstrated effective for different types of cancer cells (Rosenthal, Sostaric et al. 2004). Tumor cytotoxicity can be caused without damaging normal tissues by low intensity ultrasound combined with sonosensitizers (Dolmans, Fukumura et al. 2003). The therapeutic mechanism of SDT is generally understood by the production of ROS of sonosensitizer under the action of sound pressure (Li, Liu et al. 2012). However, the mitochondrial effect in SDT-induced cell death remains to be elucidated. In detail, selective mitochondrial autophagy, which is termed as “mitophagy”, may be key to the cell death (Youle and Narendra 2011). Nevertheless, the operating principle of mitophagy in SDT is not entirely clear. In this study, treatment of MCF-7

cells with ALA-SDT, MTT test and PI/Annexin test has evaluated cell viability and apoptosis respectively. Confocal immunofluorescence microscopy is applied to detect mitophagy. Western blot and confocal immunofluorescence microscopy are used to detect mitochondrial dynamics and redox balance respectively. Parkin knockdown was applied to assess the mitophagy role in ALA-SDT-induced cell death. It is found that through mitochondrial depolarization and fragmentation and ALA-SDT could cause mitochondrial dysfunction and induce mitophagy. Furthermore, the signaling pathway depending on Parkin takes part in ALA-SDT-induced mitophagy and plays a “Protective umbrella” in killing cells induced by ALA- SDT. In the end, it is found that excessive production of ROS initiates mitophagy. In conclusions, ROS, the product of 5-ALA-SDT could result in mitophagy mediated by PINK1/Parkin. Mitophagy mediated by PINK1/Parkin may have a protective effect on 5-ALA-SDT-induced MCF-7 cell death.

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Publications arising from the thesis

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[2] Song, L., Huang, Y., Hou, X., Yang, Y., Kala, S., Qiu, Z., ... & Sun, L. (2018). PINK1/Parkin-Mediated Mitophagy Promotes Resistance to Sonodynamic Therapy. Cellular Physiology and Biochemistry, 49(5), 1825-1839.

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Chapter 1 Introduction

1.1 Cancer

Cancer refers to related diseases in which some cells in body start to uncontrollably divide and invade into tissues that surrounded with them (Beahrs and Henson 1992). It involves trillions of cells that can appear in almost everywhere in human body. Normally, cells grow and divide into new cells in response to the needs of our body. New cells replace old or unhealthy or damaged cells after they die though necrosis, apoptosis or autophagy (Lemasters, Nieminen et al. 1998). During the development of cancer, the normal orderly procedure is destroyed. Cells may be abnormal, and some old or damaged cells that should be apoptotic or dead are not able to metabolize normally but continue to divide new cells. These uncontrollable cells grow endlessly and could grow up into tumors. Most cancers develop into solid tumors (Therasse, Arbuck et al. 2000), and there are also some cancers that do not constitute solid tumors, just like leukemia (Nakao, Yokota et al. 1996). Tumors that become cancerous are malignant, implying they will invade tissues nearby. In addition, with the growth of these tumors, some cancer cells

can cease abruptly and trip far off within the body by the blood or the bodily fluid system and produce a new tumor different from the original tumor (Sobin and Fleming 1997). Benign tumors don't unfold into adjacent tissues, which is totally different from malignant tumors. Benign tumors could be very giant, but they sometimes don’t grow back if they are removed, while malignant tumors typically could recur. Most benign tumors do not pose a threat to life (Liberman, Bonaccio et al. 1996).

The biggest difference between cancer cells and normal cells is that cancer cells are aggressive and not regulated by the body while traditional cells are not. One necessary distinction is that cancer cells are much larger than traditional cells. Unlike traditional cells, cancer cells do not grow into different cell varieties with specific functions. This can be one reason that, in contrast to traditional cells, cancer cells can continuous divide with no end. Cancer cells can escape signals that normally warn cells to prevent division or apoptosis used by the body to remove non-essential cells (Ouyang, Shi et al. 2012). In order to meet their own needs, cancer cells can affect traditional cells, molecules and blood vessels, creating a microenvironment that is in line with their own growth (Jain 2005, Joyce and Pollard 2009, Weis and Cheresh 2011). For example, cancer cells can induce blood vessels in the vicinity of traditional cells, providing the tumor with the elements and nutrients needed

for growth, and metabolize tumor waste (Nagy, Chang et al. 2009). Cancer cells can often evade the immune system by some mechanism (Dunn, Bruce et al. 2002). The body's immune system is like the "Guardian", which is organized by the organs, tissues and cells of special network, protecting the body from infections and the impact of different environments. Under normal circumstances, damaged or abnormal cells can be removed through the body's immune system, but some cancer cells can escape its effects. The immune system can even protect tumor survival and growth (Khong and Restifo 2002). With the help of some immune system cells the body's immune system can release some cells or some kind of signal to control the immune response cancer cells can actually prevent this system from killing themselves.

Cancer could be a gene-related disease, that means, the changes of genes induce the occurrence of cancer, for they manage the way our cells perform, particularly the way cancer cells grow and divide (Kaghad, Bonnet et al. 1997, Wang, Stepaniants et al. 2008). The changes that lead to cancer genes may come from our ancestors. Genetic changes will additionally increase the risk throughout one’s lifespan as errors occurs when cells divide or the harm to DNA when exposed to the environment. Environment could induce cancer include some substances and radiation, such as chemicals in tobacco smoke or external ultraviolet rays, etc. (Hill 1965, Moore and Chang

2010). Each individual's cancer features a distinctive combination of genetic changes. Further changes can occur with continuous growth of cancer. Even inside the identical tumor, distinct cells might have different genetic changes (Reya, Morrison et al. 2001). Generally, compared with traditional cells, cancer cells have a lot of genetic changes, like mutations in DNA. Some of these changes may be the results of cancer and do not play any role in cancer (Vogelstein and Kinzler 2004). Three main forms of genes are likely to be affected by the genetic changes that contribute to cancer—DNA repair genes, tumor suppressor genes and proto-oncogenes (Sherr 1996, Knudson 2001). Mutations encoded by these genes are often referred to as the "engine" of cancer. Proto-oncogenes are concerned in traditional cell growth and division. Once these genetic variants are more active than conventional gene, they will develop into oncogenes, permitting cells remains growing and alive. Tumor suppressor genes are related to dominant cells growth and division. Cells with some changes in the suppressor genes might grow out of control. DNA repair genes are concerned in repairing broken DNA. Among these genes, the mutant cells are often having alternative mutations in other genes. In summary, these mutations may cause cancer. As scientists have a lot of research to do to find out changes in certain molecules can lead to cancer, they need to explore different types of cancers that usually have different mutations. Because of

this, cancer is often characterized by the type of genetic change that drives them. Not only the parts they grow in the body, but also the shapes of cancer cells under the microscope (Kim, Ahn et al. 1996, Kimura, Nikiforova et al. 2003).

Metastatic tumor cells irrespective of the primary tumor during the transfer, it can move in the blood system. Metastatic tumors form at the location of non-primary tumors. Pathological process of metastatic cancer is cells refers to the situation that cancer cells have unfold from the original place and began to invade another place within the body. The method by which cancer cells invade alternative components of the body is termed as metastasis. Metastatic cancer belongs to the same sort of cancer cells and shares the identical name. For instance, liver carcinoma spreading to and forming a tumor within the respiratory organ is a pathological process liver carcinoma, not a respiratory cancer. Metastatic cancer cells are usually the same type of cells as the primary tumor cells (Chambers, Groom et al. 2002). Additionally, Cells of metastatic cancer usually have homology and appear to have some common molecular choices with primary tumor cells. Treatment could extend the lives of some patients. In general, treatment of metastatic cancer is a prerequisite for controlling the spread of the tumor or alleviating the

symptoms caused by it. Pathological procedural metastatic tumors can cause serious damage to the body's function (Cleeland, Gonin et al. 1994).

It is not that every change in body tissues is cancer. Observed by the microscope, the hyperplasia cells and the tissue look similar with traditional cells. Many factors or conditions, as well as chronic irritation lead to hyperplasia. Although some samples of tissue changes aren't cancer, in some tissue changes, once cells among a tissue divide faster than traditional and further cells accumulate or proliferate, hyperplasia could transform into cancer if it's not treated (Reya, Morrison et al. 2001). Due to conjoint accumulation of additional cells, dysplasia may be more serious than hyperplasia. Nevertheless, these cells appear abnormal and change in the tissue. Generally, if the cells and tissue look like more abnormal, they have more chance to be classified as cancer (Berry, Coffey et al. 1984). A more serious situation is carcinoma in situ. Although it's usually known as cancer, carcinoma in situ isn't cancer due to the abnormal cells don't invade the far side of the original tissue. Therefore, carcinoma in situ does not break through the basement membrane and invade the adjacent tissue. However, some carcinomas in situ should be treated aggressively as they may develop into cancer (Holland, Stekhoven et al. 1990, Poller, Barth et al. 1995).

There are over one hundred varieties of cancer. Varieties of cancer are typically named after the organs or tissues in which they are found. For example, lung cancer comes from cells of the respiratory organs, while brain cancer originates from cells in the brain. The following is some certain types of cancer:

Carcinomas are the foremost common form of cancer (Sørlie, Perou et al. 2001). They start from epithelial cells. Regularly, many varieties of animal tissue cells, have a column-like form underneath a microscope. Originated in a variety of epithelial cell cancer have a clear name: Adenocarcinoma is a cancer shapes in animal tissue cells that secrete fluids. Tissue invaded by epithelial cell are typically referred to as organ tissue. Most colon, breast and prostate cancers are classified as adenocarcinomas (Bhattacharjee, Richards et al. 2001). Basal cell carcinoma may originate from the underlying or basal layer of the epidermis which may be the outer layer of the human skin (Gorlin 1987).

Squamous cell carcinoma forms in a lower place on the outer skin surface. Squamous cells additionally dwell in several alternative organs, together with the lungs, bladder, abdomen, intestines, and kidneys. Beneath a microscope, squamous cells look as flat as fish scales (Prince, Sivanandan et al. 2007). Epithelial cell carcinomas are typically referred to epidermoid

carcinomas. Transitional epithelial tissue (urothelium) refers to transitional cell cancer possibly formed during a form of animal tissue. This kind of tissue formed from several layers of animal tissue cells may be larger and smaller. The tissue can be found within the linings of the ureters, bladder, and a part of the kidneys (renal pelvis), and some alternative organs, comprising a number of transitional cell carcinoma of the bladder, kidney and ureter cancer (Rheinwald and Beckett 1981).

Sarcomas are cancers of bones and soft tissues as well as muscles, fats, blood vessels, pyloric blood vessels, and plant tissues such as tendons and ligaments (Hussein and Smith 2005). The most common type of bone cancer is osteosarcoma (Ottaviani and Jaffe 2009). The most common soft tissue malignant diseases are sarcoma, Kaposi malignancy, malignant fibrous histiocytoma, dermatofibrosarcoma protuberans and sarcoma (Friend, Bernards et al. 1986).

Leukemia begins with the bone marrow tissue that produces blood. It is a cancer of the blood system which is different from solid tumors (Greaves 1988). A large number of abnormal white blood cells, called leukemia cells, in the blood and bone marrow, is similar to the condition of traditional blood cells. A small amount of traditional blood cells will increase the durability of the body, transport oxygen to its tissues, control bleeding, or fight infection.

Cancer which in lymphocytes is lymphoma. lymphocytes are white blood cell resisting disease, and they belong to immune system (Harris, Jaffe et al. 1994). In lymphoma, Abnormal lymphocytes accumulate in lymph nodes, lymph, and various body lymphoid organs. Lymphoma has two main styles: Hodgkin lymphoma – individuals who get this disease have Reed-Sternberg cell (abnormal lymphocytes). These cells sometimes generate from B cells. Non-Hodgkin cancer – this cancer can be an oversized cluster of cancers start with lymphocytes. Non-Hodgkin cancers might generate from B cells or T cells. They can grow quickly or slowly.

Multiple myeloma, starting from plasma cells, is a part of plasma cell myeloma and Kahler's disease. Abnormal plasma cells (myeloma cells) accumulate in the bone marrow and form tumors in the body's bones. They are also immune cells.

Melanoma originates from the cells that develop into melanocytes, a specialized cell that create animal pigment (pigment providing skin with color). Melanoma usually grows on the skin, but it can also appear in other pigmented tissue, like the eyes (Balch, Gershenwald et al. 2009).

There exist different kinds of brain and spinal cord cancers. These cancers are named according to the cells they originally formed or the cells

that the tumor initially formed in the central system. For example, an astrocytic tumor grows from astrocyte, which is a kind of cell supporting for the health of nerve cells. Brain tumors have two different categories, which are benign (not cancer) and malignant (cancer) (Kopelson, Linggood et al. 1980, Epstein, Farmer et al. 1992).

1.2 Cancer Treatment

Cancer has different treatments. The class of treatment is determined by the type of cancer and the stage of it. Some people with cancer only has one treatment like surgery, radiotherapy, chemotherapy. However, a lot of patients have a mix of treatments, like surgery with medical aid or radiation. Immunotherapy, targeted therapy, or hormone therapy also provide great effect in curing cancer.

Surgery refers to the process by which a doctor removes cancer from the body. Surgeons have special guidance in surgery. Surgeons often use scalpels to cut body tissue. Throughout the procedure, surgeons have special guidance in surgery. Surgery usually involves cutting the skin, muscles and bones. These incisions are often painful after surgery and require time to recover (Heald, Husband et al. 1982). Surgical process could be a form of treatment with extreme cold temperature. Cryogen or argon gas is employed

to the abnormal tissue. And the abnormal tissue will lose its function. The cold surgical process can treat early-stage skin carcinoma, metastatic tumor, and malignant neoplasm growths on the skin and cervix. The cold surgical process is additionally known as cryotherapy (Hewitt, Zhao et al. 1997).

Lasers treatment is an accumulated beam which can be applied to pierce the tissue and cure the target area. Lasers could focus accurately on little areas, so lasers treatment is often applied in precise surgeries. The growth of some exotic organisms can be prevented with a laser (Grevelink and Byers 1997). clinically, lasers are commonly used to destroy surface tumors that grow in the body or internal organs, for example, cervical, vaginal, esophageal, basal cell carcinoma, and non-small cell lung cancer.

Radiotherapy could be a treatment to cure cancer by which high doses of radiation is employed to eliminate the cancer cells and lessen tumors (Clarke, Lê et al. 1985). With this therapy, cancer cells can be killed, or their growth can be slowed down by destroying their deoxyribonucleic acid. If deoxyribonucleic acid is broken beyond repair, the dividing of cancer cells will be ceased, or the death of cancer cell can be induced. Once the broken cells die, they're softened and removed by the body. Therapy doesn't kill cancer cells immediately. Days or weeks will be taken before deoxyribonucleic acid is broken enough for cancer cells to die. When therapy

ends, it also takes weeks or months for cancer cells keep dying (Garcia-Barros, Paris et al. 2003). External beam and internal ones are two main therapy (D'amico, Whittington et al. 1998, van de Bunt, Van der Heide et al. 2006, Chofor, Harder et al. 2012). In external beam therapy, a massive and noising machine emit radiation to cancer cells. The radiation beam moves around the body, emitting radiation to part of the body from several directions. External beam therapy could be a native treatment, which suggests it treats a selected part of the patient. Take the cancer in the respiratory organ as an example, it'll apply radiation solely to the chest, not the whole body. Internal therapy could be a treatment during which a supply of radiation placed within the body. The radiation supply is solid or liquid (Ozhasoglu and Murphy 2002).

Chemotherapy could be a sort of cancer treatment with which medicine is used to cause cell-death of the cancer. It prevents or slow the expansion of cancer cells; cancer cells grow and divide rapidly (Dorr and Fritz 1980, Peters III, Liu et al. 2000). Chemotherapy is employed to cure cancer, reduce the possibility of cancer relapse, stop or slow cancer growth. It can also ease cancer symptoms through shrinking tumors that are inflicting pain and alternative issues. In addition to chemotherapy, it can not only kill invasive cancer cells, but also affects the expansion of fast-growing and dividing healthy cells, such as cells that grow in the mouth, intestines and hair follicle.

The injury to healthy cells could cause facet effects, like mouth sores, nausea, and hair loss. These side effects usually recover after the therapy is completed (Lindley, McCune et al. 1999).

Hyperthermia is a treatment using high heat to focus on tiny areas of body tissue. Under the high heat, cancer cells can be harmed or killed or be more vulnerable to radiation and other chemotherapy drugs (Vernon, Hand et al. 1996, Wust, Hildebrandt et al. 2002). Radiofrequency ablation is one form of physiological state in which high-energy radio waves are used to generate heat. Radiofrequency ablation have not been widely used in research and clinical trials (HYPERTHERMIA 1992).

Photodynamic therapy could be a form of treatment using medicine that in response to a particular form of light-weight. Once the cancer tissue exposes in the current light-weight, these medicines will become active and kill near cancer cells. Photodynamic therapy is frequently employed to treat or alleviate symptoms caused by carcinoma, zymosans fungoides, and non-small cell carcinoma (Dougherty, Gomer et al. 1998, Dolmans, Fukumura et al. 2003).

1.3 Ultrasound bio-effect and Sonodynamic therapy

Ultrasound refers to a mechanical wave which vibrates periodically at a frequency equal to or greater than 20 kHz in an elastic medium without stopping. In the liquid, the speed is about 1000 to 1600 m /s, which means that the wavelength range is from microns to centimeters (O'Brien Jr 2007). Therefore, the acoustic field cannot be coupled to the molecular strength level , such as the organic milieu on the molecular level. There is no harm for DNA, RNA and protein in normal physiology condition. Therefore, ultrasound is the safest, and has an excellent tissue penetration. From a clinical perspective, this is a very attractive feature and has led to huge meaning of the ultrasound of clinical function. Ultrasound has been used for diagnostic imaging of smooth tissue (Szabo 2004, Wein, Brunke et al. 2008). The different treatment options relate to thermal effects, since the absorption of ultrasound hyperthermia (the tissue temperature was maintained at 40-45 °C.) and a thermal ablation (tissue temperature 60 to 85 °c, a few seconds) (Hynynen, Vykhodtseva et al. 1997, Vykhodtseva, Sorrentino et al. 2000). The medical implication of ultrasound includes the enhancement of chemotherapy, surgery that isn't invasive including necrosis of stable tumors, vascular closure and correction of cardiac arrhythmias (Kremkau 1979).

As we all tend to recognize that cancer has become one of the main reasons for depriving human life, anti-tumor medicine has attracted the attention of many researchers in the world. But all types of cures have their own limitations, posing a serious challenge for cancer medical care (McNicol, Horowicz-Mehler et al. 2003, McNeely, Campbell et al. 2006, Lemieux, Maunsell et al. 2008). Surgical treatment has problems in eliminating cancer cells and does not cure metastatic growth. Chemotherapy and radiation therapy will kill some cancer cells, but it will also damage the normal tissue. More importantly, cancer cells may develop tolerance in long-term treatment and radiation therapy, which is the biggest problem in cancer therapy. Immunity therapy is a new and effective cancer treatment, but it is overpriced and can lead to immune system storms. Therefore, to find an effective, safe and inexpensive method of treatment is imperative.

Sonodynamic (SDT) has evolved into a completely unique and promising non-invasive method from photodynamic therapy (PDT) (Dougherty, Gomer et al. 1998, Dolmans, Fukumura et al. 2003). In the early 1980s, many hematoporphyrin derivative (HPD) applied in PDT. Once it is activated by ultrasound, it may cause cell damage (Chaudhuri, Keck et al. 1987). Since then, it is indisputable that many newly born HPDs are likely to be applied as sonosensitizers. The SDT is different from PDT, the sensitizer

of SDT is activated by ultrasound (Tang, Liu et al. 2008, Tang, Liu et al. 2009). PDT has a short penetration depth and is not effective in treating deep tumors. However, the mechanism of the sonodynamic effect is the production of reactive oxygen radical (ROS), when the low-intensity ultrasound cooperates with the sensitizer (Rosenthal, Sostaric et al.). The advantage of SDT is that ultrasound can concentrate in soft tissue, penetrating dozens of centimeters. This just makes up the lack of PDT. Therefore, SDT is a very exciting treatment that produces amazing anti-tumor effects in vitro and in vivo study (Li, Song et al. 2008).

The cooperation of ultrasound and sonosensitizers makes the SDT work. Under suitable conditions of ultrasound, the collapse of gas-filled bubbles can form cavitation (Kremkau, Kaufmann et al. 1976). There are mainly two kinds of stable and unstable cavitation. Stable oscillate bubbles make streaming of the encircling liquid which ends up in a mixture of the encircling media, while the air bubbles in the unstable method of cavitation will expand to a resonance size and explode before they collapse violently. (Frulio, Trillaud et al. 2010).

Under the action of this acoustic chemical reactor, the acoustic sensitizer attached to the surface of the growing cell will be activated into excited state, once it touches the ultrasound. After the activated sonosensitizer turns back to the basic state, the free energy is then transferred to the

surrounding oxygen in order to provide a certain amount of ROS. The ROS has direct cellular toxicity. Konno et al. reported that although HP-treated cells were proved causing no mechanical cavitation, they think the cells were sensitive at low intensity of ultrasound. It is worth noting that the ultrasonic intensity used in this study produced sonoluminescence through stable cavitation. Yasuda et al. are very certain that cavitation could contribute to the generation of ROS (Konno, Watanabe et al. 2003, Yasuda, Yoshizawa et al. 2015, McHale, Callan et al. 2016).

Sonoluminescence refers to the light emitted by ultrasound caused cavitation on the cell surface. (Yin, Chang et al. 2016). Umemura et al. researched the emission in salt solutions with that light and activated acoustic sensitizers such as HP in similar conditions as PDT. (Umemura, Yumita et al. 1990). Sazgarnia et al. verificated sonoluminescence in the phantom of the gel base through the combination of the original protoporphyrin IX (PpIX) with gold nanoparticles, with ultrasound radiation at a frequency of 1.1 MHz The gold nanoparticles acted as nucleation centers for cavitation in the study and they detected the sonoluminescence signals at 350–450 nm, 450–550 nm and 550–650 nm (Sazgarnia, Shanei et al. 2013).

SDT has been proved to induce the production of ROS through mechanical cavitation under sonoluminescent light. This can be applied to

manufacture of microbubbles in water containing environment, for example, cytoplasm. Once microbubbles collapse, a lot of energy is generated, so they start emitting sonoluminescent light, and then produce ROS. The microbubbles will cause serious damage to cells because of the energy released by implosion. The shear force through fluid mechanics can be applied in destroying malignant cells. The structures of the cells are destroyed in that condition. Cheng et al. are certain that PpIX induced by 5-aminolevulinic acid (5-ALA) is mainly harmful to the mitochondria in the cells. It produces too much ROS in mitochondria, causing THP-1 to clear cell necrosis. (Cheng, Sun et al. 2013, Chen, Zhou et al. 2014).

By absorbing the low energy of ultrasound, sonosensitizers in the tumor area convert oxygen into a variety of extremely active oxygen, such as one singlet oxygen, which directly causes irreversible damage to cancer cells. Chen et al. studied mice bearing SPCA-1 which were exposed to the ultrasound, with or without chlorin e6 (Ce6) (Cheng, Sun et al. 2013). The results indicated that concentration of the Ce6 in tumor tissue was significantly higher than the normal tissue around the tumor. In vivo, ultrasound and Ce6 couldn't hurt tumor cells respectively, but the combination of them greatly hindered the growth of tumors. Flow cytometry

proved that Ce6-mediated sonodynamic therapy could function well because ROS could lead to cell necrosis.

After SDT triggers an increase in ROS levels in cells, the mitochondrial membrane is damaged by lipid peroxidation. In this case, mitochondrial membrane potential depolarized and mitochondrial membrane permeability increases consequently (McEwan, Owen et al. 2015). To obtain the best parameters, Sun et al. studied an experimental system during ultrasound irradiation to observe the ROS and mitochondrial membrane potential (MMP) loss in real time (Sun, Xu et al. 2015). Macrophages derived from THP-1 were cultured with 5-ALA and processed under different intensities of ultrasound. During ultrasound treatment, once the ultrasound intensity is 0.48 w/cm^2, the ROS elevation and MMP loss in THP-1 derived macrophages can be determined. Recent reports suggest that intracellular ROS can be used to mediate cytotoxic effects in SDT (Hachimine, Shibaguchi et al. 2007).

Damage to the mitochondrial membrane may cause the release of cytochrome (Cyt C) from mitochondria to the cytoplasm, and lead to future activation of procedural cell death pathways dependent on caspase. Su et al. found that PpIX-mediated SDT would lead to procedural cell death and the autophagy of leukemia K562 cells (Su, Wang et al. 2015). Under morphological observation and biochemical analysis, the procedural cell

death of mitochondrial dependence was identified. Additionally, SDT has been shown to induce K562 cells to autophagy. A similar study investigated the procedural death behavior of U937 cells, which is caused by SDT mediated through HP monomethylether (HMME). In comparison with ultrasound group, HMME group and the control group, the ROS content in cells increased greatly when combined with SDT. Hao et al investigated the effect of intracellular calcium overload on programmed cell death of C6 brain tumor cells by SDT mediated through HMME. The results indicated that in the media of enough Ca^{2+}, SDT makes the intracellular ROS increased, the MMP reduced, and Cyt C released from mitochondria. In short, intracellular ROS growth is directly related to the increase of the concentration of Ca^{2+}, so apoptosis has improved (Su, Wang et al. 2013, Hao, Song et al. 2014).

In conclusion, SDT can make non-invasive elimination of tumors possible. The high therapeutic effect of SDT in cancer is endowed by its powerful functions. Several studies have demonstrated that a large quantity of intracellular ROS assembled by SDT will produce direct toxicity in cancer cells (McCaughan, Rouanet et al. 2011). Recently years, many studies have also shown that SDT has a regulatory effect on the cancer microenvironment (McCaughan, Rouanet et al. 2011). Inhibition of the effects on growth vessels, which causes the growth of immunosuppressive tumors to expand. The

mechanism that ultrasound activates sensitizer trigger effect for cancer is widely accepted. SDT acts mainly through the induction of high intracellular ROS, which promotes lipid peroxidation and damages the mitochondrial membrane, depolarizing the mitochondrial membrane potential, thereby increasing the mitochondrial membrane porosity (Sun, Xu et al. 2015).

1.4 Mitochondria and Mitophagy

Apart from supplying energy to cancer through glycolysis, mitochondria is also important signaling platform in tumor progression. (Zong, Rabinowitz et al. 2016). As dynamic organelles, mitochondria change their numbers and shapes incessantly in the process of fission and fusion. Maintaining mitochondrial morphology requires balancing mitochondrial fusion and division, which is accomplished by its fusion and cleavage proteins (Scatena 2012, Vasquez-Trincado, Garcia-Carvajal et al. 2016). It is known that the balance between fusion and fission plays a key role for the whole cellular health. Normally, mitochondria typically exhibit a tubular or reticular shapes. However, they endure a perinuclear aggregation that suffers mitochondrial division and mitochondrial autophagy when exposed to physiological stress. Under normal conditions, dysfunctional mitochondria are separated into low membrane potentials by cleavage events and

subsequently degraded by autophagosomes. Mitochondrial dysfunction is thought to cause mitochondrial autophagy (Scott and Youle 2010, Youle and van der Bliek 2012, van der Bliek, Shen et al. 2013).

Mitophagy is a catabolic regulates process (Saito and Sadoshima 2015). In mitophagy, the dysfunctional or impaired mitochondria of cells are degraded by themselves to keep functional status of mitochondrial population. Thus, mitophagy can reduce cell damage and facilitates cell homeostasis and survival. The lack of mitophagy triggers inflammation and cell death and is closely related to many neurodegenerative diseases such as Alzheimer's disease (AD) or Parkinson's disease (PD). The Parkin signaling pathway and putative kinase 1 induced by PTEN (PINK1) makes an important impact in mitochondrial autophagy (Kubli and Gustafsson 2012, Wu, Xu et al. 2015, Zhang, Mi et al. 2016). It is responsible for triggering impaired mitochondria for recognize autophagy selectively. The level of PINK 1 in normal mitochondria is kept at a very low level. And it is introduced constructively on the mitochondrial membrane and degrades immediately. However, when the mitochondrial membrane potential disappears, PINK 1 stabilizes on the outer membrane, forming a large complex on its surface, which absorbs Parkin into the damaged mitochondria. Briefly, mitochondrial depolarization results in the expression of PINK 1 at high levels. And Parkin is subsequently

absorbed into the mitochondria. This unique Parkin PINK 1 and may be selectively adjusted and effectively replace damaged mitochondria (Geisler, Holmstrom et al. 2010, Kawajiri, Saiki et al. 2010, Wu, Xu et al. 2015).

Numerous evidence demonstrate that reactive oxygen species (ROS) is conducive to mitochondrial dysfunction and apoptosis. It has been shown that ROS can cause apoptosis and cell death (Trachootham, Lu et al. 2008, Circu and Aw 2010). Intracellular oxidative stress mainly comes from and targets at mitochondria, which makes mitochondria particularly sensitive(Su, Wang et al. 2015, Trendowski 2015, You, Deepagan et al. 2016). Typically, cellular redox homeostasis ensures an appropriate response under pressure. Cells in both the cytoplasm and mitochondria antioxidant system comprise a free-radical scavenger manganese superoxide dismutase (MnSOD) and catalase to remove excess ROS, maintaining redox homeostasis (Gonzalez-Parraga, Hernandez et al. 2003, Candas and Li 2014). However, excessive oxidative stress may cause organelle dysfunction, disrupting redox homeostasis, and ultimately, abnormal cell death.

As mitophagy plays a key role maintaining the functional status of mitochondria. It has been determined that mitophagy is involved in MCF-7 cells treated by 5-ALA-SDT. Our study further explored the role and molecular mechanisms of mitochondrial autophagy. Our research also

ascertained that mitophagy mediated related to the PINK1-Parkin mediated is able to prevent apoptosis and cell death as well as upstream effects of ROS processes. Therefore, As the targeted mitochondrial autophagy has been clinically tested and confirmed, the therapeutic efficiency of ALA-SDT has been further improved.

Chapter 2 Materials and Methods

2.1 Reagents and antibodies

Purchased from Sigma (St Louis, MO, USA), 5-aminolevulinic acid (ALA) was dissolved in PBS to a stock concentration of 1M and was stocked at -20°C away from light. Sigma-Aldrich (St Louis, MO, USA) offers paraformaldehyde, bovine serum albumin (BSA), N- acetylcysteine (NAC), diphenyl chloride (DAPI), as well as Anti-GAPDH, and anti-LC3 polyclonal antibody. Goat anti-mouse IgG- IgG-horseradish peroxidase and goat anti-rabbit IgG-horseradish peroxidase were come from Thermo Fisher Scientific. Antibodies for anti-caspase-3, anti-caspase-9 were purchased from Cell Signaling Technology (Santa Cruz, CA, USA). Human Lamp-2 antibody was obtained from R&D (Minneapolis, MN, USA). PINK1 antibody was from

Novus Biologicals (Littleton, CO, USA). Anti-Parkin antibody was purchased from Abcam (Cambridge, UK), LC3B polyclonal antibody, Catalase polyclonal antibody, MFN1 polyclonal antibody, SOD2 polyclonal antibody, and FIS1 polyclonal antibody were from Thermo Fisher Scientific (Carlsbad, CA, USA). Immunofluorescent secondary anti-rabbit/mouse IgG was from Jackson Immune Research (West Grove, PA, USA).

2.2 Cell incubation and Sonodynamic therapy

Deriving from a cell library Academy of Sciences (Shanghai), MCF-7 cells were stored in Dulbecco's modified Eagle medium (DMEM, Life Technology, Carlsbad, USA). It was added with 10% fetal bovine serum (FBS, Life Technology), 100 units / ml penicillin and 100 ug / ml streptomycin. At a temperature of 37 ℃, MCF- cells were stored in 95% air and 5% CO_2 humidified chamber. MCF-7 cells were digested with 0.25% trypsin and passaged at 80% confluence. Randomly, MCF-7 cells were classified into four groups: (1) control, (2) ultrasound alone (ultrasound), (3) ALA alone, and (4) ALA plus ultrasound (SDT). 1mM ALA was used to incubate cells in ALA and ALA-SDT groups. And 4h was taken to load drug in each group with DMEM medium containing 10% FBS. The cells of the ultrasound group and

the SDT group were placed under an ultrasonic wave with an intensity of 0.25 W/cm^2 at a frequency of 1.0 MHz burst mode with the pulse repetition frequency of 100Hz and duty cycle of 10% for 10 min. The ultrasonic generator and a power amplifier used in this study were purchased from Tektronix (Oregon AFG 3251) and AR Inc (Model 500A250C). The planar transducer has a diameter of 35 mm. For irradiation, an ultrasonic couplant was used to fill the spacing between the transducer and the cell culture plate. By this way, transmission of ultrasound can be promoted. After the treatment, cells used for different analyses were incubated in fresh medium for different times (2 hours, 4 hours, 12 hours).

2.3 Determination of cell viability

Cell viability was determined at various time points after ALA-SDT treatment using Cell Counting Kit-8 (Sigma-Aldrich) with reference to the manufacturer's guidelines. Our method is to incubate the cells with 96-well plates at a density of 5000 cells per well. The cells are incubated in 100-μl medium for 24 hours. And then CCK-8 reagent 10μ1 per well at 37 ° C and 5% CO_2 were added. Cytotoxicity was measured in 1 hour. At 450 nm, the absorbance of the sample relative to the blank control was determined as the

detection wavelength. Compared with the control group, changes in cell viability were observed.

2.4 Apoptosis assay was detected by flow cytometry.

Being bred at a density of 5×10^5 cells in 6-cm dishes, cell culture time was 24h. Referring to the manufacturer's guidelines, cell apoptosis at various time point after ALA-SDT was measured by Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher Scientific). After being collected, cells were incubated with 1 μL of the PI working solution and 5 μL of the annexin V conjugate for 15 minutes at room temperature. And then it will be analyzed through the BD Accuri C6 Software (Becton-Dickinson, USA) and FACS Calibur flow cytometer.

2.5 Detection of mitochondrial membrane potential

JC-1(Sigma-Aldrich) was utilized to determine mitochondrial membrane potential. Briefly, before being washed with FACS buffer, at 37 ° C cells were dark staining with 2.5μM JC-1 for 30 min. FACS Calibur flow cytometer and BD Accuri C6 Software (Becton-Dickinson, USA) are responsible for analyzing the data. Excitation and emission settings JC-1 monomers were 488 nm and 515 ~ 545 nm (FL1 channel); excitation and

emission settings JC-1 aggregations were 488nm and 564 ~ 606nm (FL2 channel).

2.6 ATP generation test

Consistent with the manufacturer's instructions, firefly luciferase-based ATP assay kit was utilized for measuring cellular ATP production (Thermo Fisher Scientific). After lysis centrifugation for 5 min at 12000 g, the 10µ1 cell lysate and 100µl ATP detection fluid were mixed in 96 well white plate. These data are coordinated by a microplate reader Biotek (Biotek Tools Inc., Winooski, Vt, USA) collection. Protein concentration in the cell lysate was measured by BCA kit. Data was evaluated using by ratio of cellular ATP levels and protein concentration.

2.7 Mitochondrial dynamic assay

Consistent with the manufacturer's instructions, mitochondria were tracked by CellLight Mitochondria-GFP (Thermo Fisher). In a word, the reagent was involved in the incubation with cells all the night, mitochondria-GFP behavior can be traced in live cells. After being treated by ALA-SDT,

cells were observed using laser-scanning confocal microscopy with X63 objective (Nikon, Tokyo, Japan).

2.8 Immunofluorescence microscopy of mitophagy

According to the standard guidelines, mitochondrial autophagy and lysosomes colocalization are the determining factors of mitophagy (Ding and Yin 2012). Each 35mm confocal culture dish (SPL Life Sciences, Korea) was inoculated with 1 x 10^5 cells for 24 hours. 100 nm Mito Tracker Deep Red stains cells (Thermo Fisher) for 15 min at a temperature of 37°C. Then, PBS was used to wash cells and 4% PFA was utilized to fix them for 15min at room temperature. After being fixed, another night was taken for further incubation. Antibodies against autophagosome marker LC-3 or LAMP 2 and Alexa Fluor 488- or 594-conjugated secondary antibody will be added. 5% bovine serum albumin (Sigma) was used to dilute the antibodies. The nuclei were dyed for 10min at room temperature. Laser-scanning confocal microscopy with X63 objective (Nikon, Tokyo, Japan) was used to visualize samples. Under each condition, 20 to 30 cells were captured. The number of mitochondria colocalized with LC3 or lamp 2 was quantified.

2.9 Cell lysis and Western blot analysis

In order to get cell lysates, cold PBS was applied to wash the cells. Then,

the cells were lysed in RIPA in lysis buffer for 10 min on ice. (50mM Tris-HCl, pH 7.4, 150mM NaCl，1% NP-40，0.5% sodium deoxycholate，0.1% SDS). The buffer also contains protease inhibitor mixture (Roche, Basel, Switzerland) and a phosphatase inhibitor mixture (Roche). 10% polyacrylamide gels were loaded by the complexes which had went through the quantification and denaturation. Then the complexes moved onto polyvinylidene difluoride (PVDF) membranes. Membranes were then blocked with 5% milk diluted in PBS containing 0.05% Tween 20 (PBST) for 1 h and then immunoblotted at 4°C overnight with the specified primary antibodies. After washing in PBST for 10 min three times, the membranes was incubated 1h at room temperature with the corresponding secondary anti-rabbit/mouse IgG. After treatment of these members with ECL reagent (USA, Bio-Rad), a FluoChem E Imager (Protein-simple, USA) was used to observed the samples. Image J was applied to quantify the density of protein. The GAPDH protein is considered an internal standard for semi-quantitative.

2.10 RNA interference

Thermo Fisher offers the small interfering RNA (siRNA) duplexes. In line with the manufacturer's instructions, Lipofectamine^{TM} 2000 was used to conduct iRNA. To summarize, 1 x 10^5 cells were inoculated and incubated for

24 hours in a 35mm confocal culture dish (SPL Life Sciences, Korea). 20 μM synthesized siRNA transfected cells to target at Parkin. The siRNA and Lipofectamine^{TM} 2000 were respectively diluted in serum-free DMEM and incubated for 5 min at room temperature. The two diluted solutions were then softly mixed, incubated for 20 minutes, and finally added to the cells. Inhibition was observed after 24h after transfection, the expression of Parkin, analyzing the Parkin expression with Western blot.

2.11 Determination of cell and mitochondrial ROS amount

DCFH-DA(Sigma-Aldrich) was used to measure Intracellular ROS. In brief, MCF-7 cells were incubated with 10 mM DCFH-DA diluted with DMEM at 37°C for 20 min, and then washed with PBS for three times. The labeled cells were trypsinized and analyzed by flow cytometry. Referring to the manufacturer's instructions, the production of mitochondria-derived reactive oxygen species (mROS) is measured by MitoSOX (Invitrogen, USA). 10μM of MitoSOX were applied for the incubation of cells at 37°C for 20 min. Laser-scanning confocal microscopy with X63 objective (Nikon, Tokyo, Japan) was used to observe labeled cells which were washed with PBS three times. Finally, Nikon NIS-Elements software serve as the tool for analysis.

2.12 Statistical analysis

GraphPad Prism software was applied to do statistical analysis. Image processing is handled by Image J, Photoshop CS, and Illustrator CS software in accordance with general guidelines. All data are expressed as mean ± SEM, using a two-tailed Student's t-test or one-way ANOVA. It is considered that P-values <0.05 were of significance statistically.

Chapter 3 Results

3.1 Cytotoxicity and apoptosis were induced by ALA-SDT induces in MCF-7 cells.

A CCK-8 assay was used to measure the cytotoxicity of ALA-SDT on MCF-7 cell at various time points after SDT treatment. This result demonstrated that neither ALA nor ultrasound groups alone could induce severs cytotoxicity. And ALA-SDT significantly reduced the survival rate of MCF-7 cells to 25% and 32% at 4h and 12h after SDT (Figure 1A, $p < 0.05$). We also studied the effect of ALA-SDT on apoptosis. It was found that the SDT 2h, 4h and 12h, ALA-SDT induced apoptosis rates were 10%, 17% and 20%, 3% in the control group (FIG. 1b, $p < 0.05$). This data indicates that

ALA-SDT inhibits MCF-7 cell's growth in a time-dependent way. The experiment was performed 12 h after ALA-SDT.

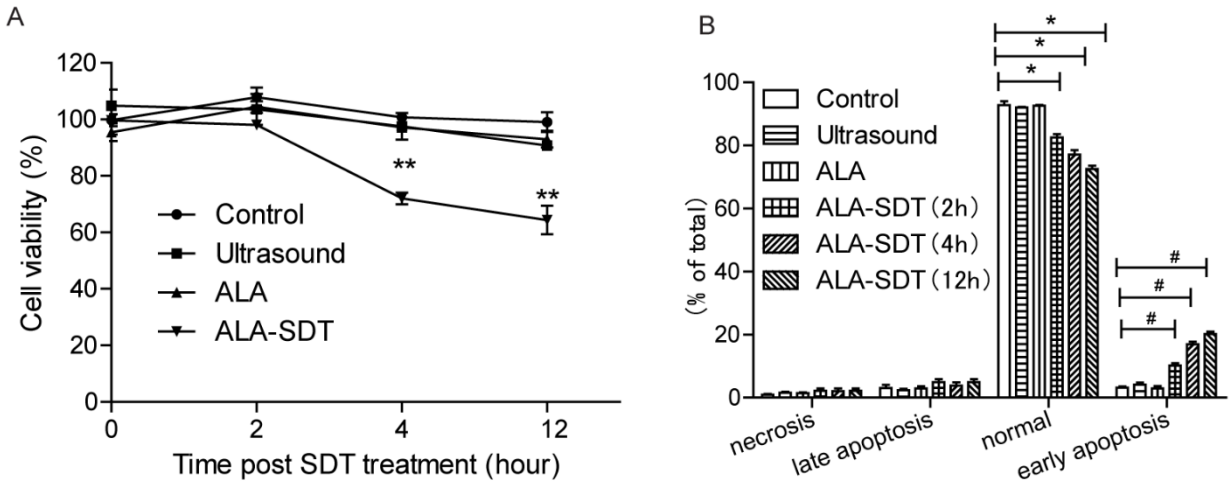


Figure 1. Cell viability and apoptosis after ALA-SDT. Cell viability and apoptosis after ALA-SDT (A) CCK-8 assay was applied to measure Killing effect of ALA-SDT on MCF-7 cells. The independent experimental data is described by mean ± sem. The curve marked with ** means ALA-SDT group is significantly different from the control group ($P < 0.01$). (B) Flow cytometry was used to detect apoptosis of membrane-v and propidium iodide (Pi) double-stained cells after ALA-SDT. The proportion of early apoptotic cells (Annexin-v/pi-) and late apoptotic cells (Annexin-v/pi) in the total number of cells were observed. Based on 3 independent experiments, the data represents the mean ± sem. Compared with the control group *$p<0.05$.

3.2 ALA-SDT causes mitochondrial depolarization and fragmentation in MCF-7 cells.

ALA-SDT treatment mainly targets at mitochondrial function. Next, we studied mitochondrial function in this study. It can be seen from Figure 2A that ALA-SDT increased the aggregation rate of JC-1 monomer from 17% in

the control group to 59% in the SDT group, indicating a sharp decline in mitochondrial membrane potential ($p < 0.05$). At the same time, the production of ATP was cut down by ALA-SDT from 11nmol/mg in control cells to 5.9nmol/mg in the SDT group (Figure 2B, $p < 0.05$). To further investigate the effects of ALA-SDT on mitochondrial dynamics, we photographed mitochondrial morphological images showing a punctate increase in short mitochondria and a decrease in tubular mitochondria in the ALA-SDT group compared to controls containing long tubular mitochondria (Figures 2c and 2d). We also examined the regulation of mitochondrial division and fusion of proteins, which helped us explore the way ALA-SDT alters mitochondrial morphology in MCF-7 cells. The results showed that ALA-SDT significantly reduced the mitochondrial fusion protein MFN 1 (Fig. 2e, quantitation in Figure 2f, $p < 0.05$). In contrast, the expression of the mitochondrial fission protein Fis 1 was significantly increased after the ALA-SDT treatment. And the expression of Opa 1 remained unchanged.

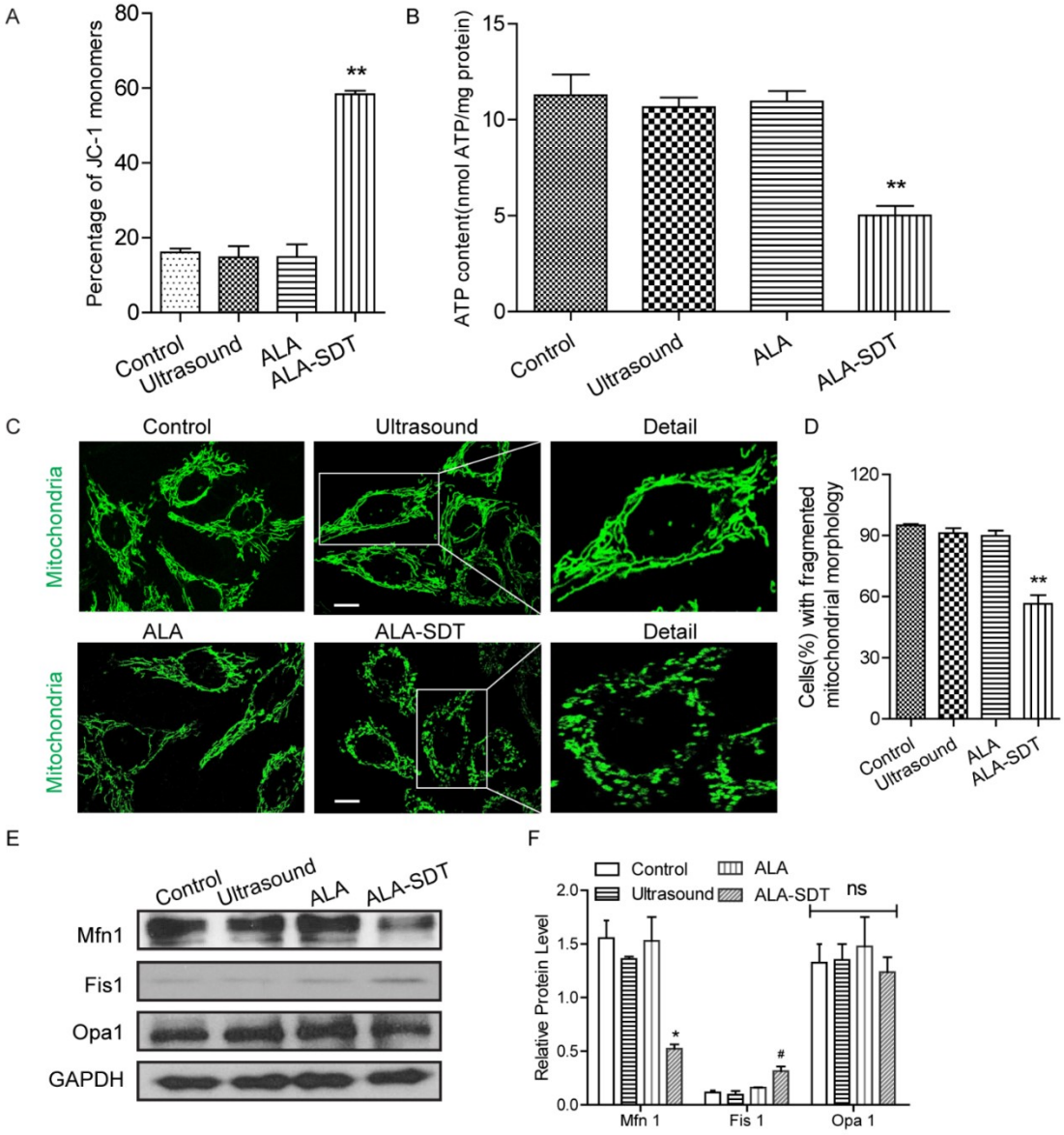


Figure 2. Mitochondrial dysfunction is ALA-SDT induced intracellular after 2h measuring mitochondrial membrane potential. (A) Cells were stained with JC-1 probe. Flow cytometry was used to collect the data. The mitochondrial potential was expressed by the percentage of JC-1 monomers. Data represents the mean ± SEM based on 3 independent experiments. **$p < 0.01$ vs. control. (B) Measures ATP production 2h after ALA-SDT treatment with an ATP determination kit. Data represents the mean ± SEM based on 3 independent experiments. **$p < 0.01$ vs. control. (C) Captured the images of GFP-tagged mitochondria when exposed to ALA-SDT in MCF-7 cells. Typical tubular mitochondria in control group and fragmented mitochondria in ALA-SDT group can be clearly seen in the enlarged images. Representative images are displayed in (C), where quantitative data is in (D). Data represents the mean ± SEM based on 3 independent experiments. (*$p < 0.05$ vs. Control). The scale represents 10μm. (E, F) a treated by ALA-SDT to access the protein expression level of mitochondrial

3.3 Mitophagy was increased in MCF-7 cells treated by ALA-SDT treated.

There is increasing evidence that mitophagy is the process by which autophagy clears damaged mitochondria, through which it maintains normal cell function. Since mitochondrial dysfunction results from ALA-SDT, we further investigated whether, in the process, mitophagy was caused. Our universal markers LC3 and lamp 2 are used for staining of autophagic vacuoles and lysosomes respectively. Yellow fluorescence analysis was performed on colocalization of mitochondria with acidic organelles (autophagosomes and lysosomes). As can be seen from Fig. 3a and Fig. 3b, ALA-SDT induced co-localization of mitochondria and autophagosomes, and the control group did not co-localize. These results can also be seen in Figures 3C and 3D: It is the co-localization of mitochondria and lysosomes, rather than the control group, that ALA-SDT resulted in. In conclusion, these results indicate mitophagy was induced after ALA-SDT treatment.

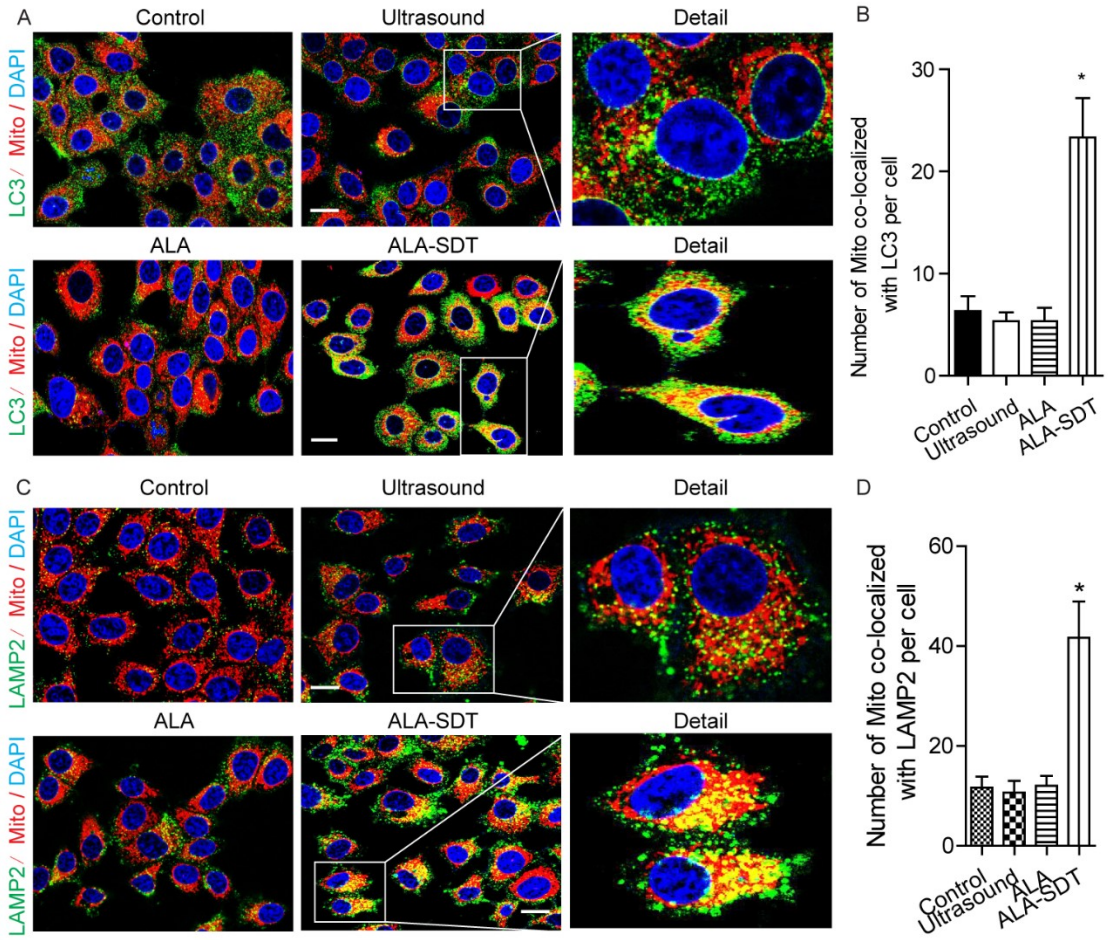


Figure 3. ALA-SDT-induced mitophagy in MCF-7 cells. (A, B) MCF-7 cells were treated with ALA-SDT. Twelve hours later, treated cells were stained with mitochondrial marker Mitotracker and autophagy marker LC3. The number of mitochondria co-localized with LC3 per cell was quantified. Each experiment to analyzed at least 25 cells. (A) displays representative images while (B) shows quantification data. Data represents the mean ± SEM of three independent experiments. *$p < 0.05$ related to control. (C, D) 12 hours after ALA-SDT treatment, applying the mitochondrial marker Mito and lysosome marker Lamp2 to stain MCF-7 cells. (C) displays representative images and (D) shows quantification data. Selecting the number of co-localized lysosomes and mitochondria from 25 cells in three experiments. The data represents the mean ± SEM. *$p < 0.05$ related to control. Scale bar represents 50μm.

3.4 ALA-SDT-induced mitophagy involves Parkin-dependent signaling pathway

The latest research shows that in related fields, PINK 1-Parkin signal is a key mitophagy control. Therefore, we subsequently researched whether the PINK1-Parkin signaling was involved in mitophagy induced by ALA-SDT. Western blot results showed that ALA-SDT increased the expression level of PINK 1 but did not increase the expression of Parkin compared with the control group (Figure 4A with quantification in Figure 4B, $p < 0.05$). Parkin location in MCF-7 cells is detected by immunofluorescence. We discovered that Parkin significantly shifted from the cytoplasm to the mitochondria in the perinuclear region in response to the ALA-SDT (Fig. 4C, quantitative see Figure 4D) when compared with the control (Figure 4C with quantification in Figure 4D). In a word, PINK1-Parkin was proved to involve in ALA-SDT. Suppression of mitophagy exacerbates ALA-SDT-induced apoptosis and cell death.

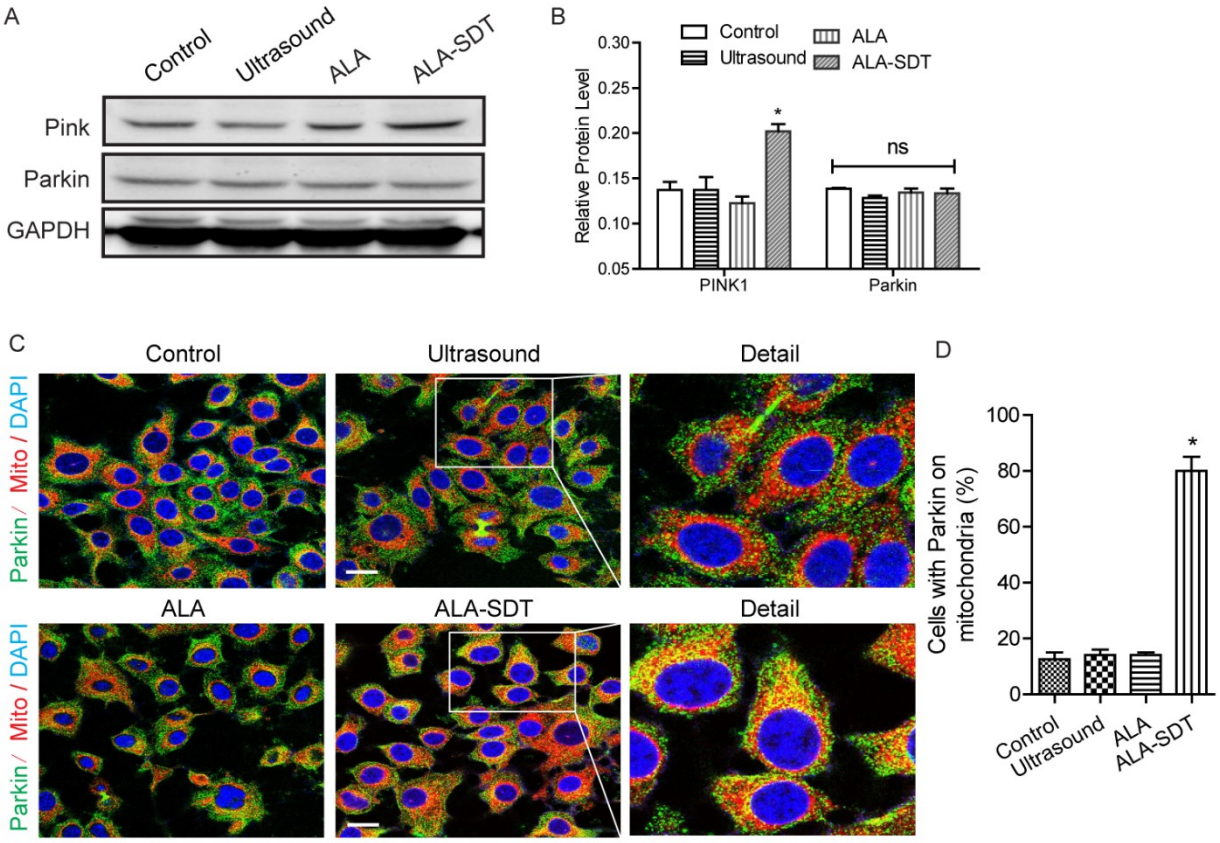


Figure 4. Mitophagy related to ALA-SDT involves PINK1/Parkin pathway. (A, B) Assessing twelve the levels of PINK1 and Parkin by Western blot several hours after ALA-SDT treatment. Figure 4A, B displays representative images of blots and quantification from three independent experiments. * $p < 0.05$, as compared with the control. ns, not significant. (C, D) the mitochondrial translocation of Parkin induced by ALA-SDT was determined by immunofluorescence. Mitotracker (red) and anti-Parkin (green) were used to stain the cells. (C) displays representative images while (D) displays quantification data. Per experiment analyzes at least 25 cells. Three independent experiments were conducted to determine the fluorescence intensity of Parkin aggregates on mitochondria. *$p < 0.05$ vs. control. Scale bar represents 50μm.

3.5 Inhibition of mitophagy aggravated apoptosis and cell death induced by ALA-SDT.

To further validate the function of PINK 1-Parkin-mediated mitophagy in ALA-SDT-induced cell death, we knocked down Parkin from MCF-7 cells

with siRNA. Figures 5a and 5b show that Parkin was silenced to 25% of its normal level after 24 h of transfection. In addition, ALA-SDT-induced mitochondrial and lysosomal colocalization of the Parkin gene knockdown were also lower (Figure 5C). The results showed that knockdown of Parkin gene can inhibit the occurrence of mitophagy. We then tested the effect of PINK 1-Parkin-mediated mitophagy on ALA-SDT-induced cell death. Figure 5e shows that Parkin gene knockdown caused cell death (20%, $P < 0.05$, $n = 5$). Parkin siRNA significantly increased the apoptotic rate by 10% compared to the mock group (Fig. 5f). This is consistent with the view that PINK 1-Parkin-mediated mitophagy is a cell death induced by ALA-SDT.

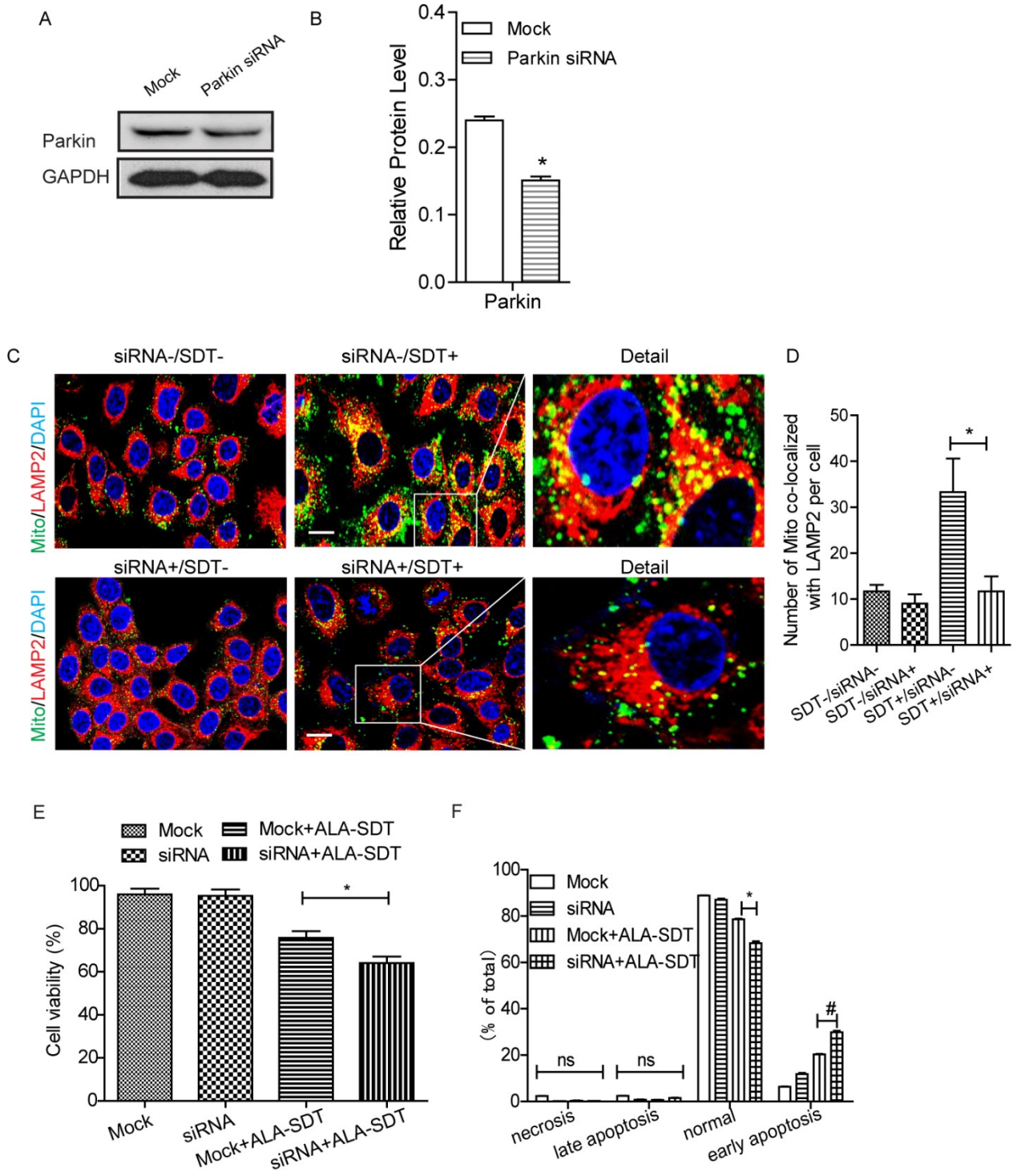


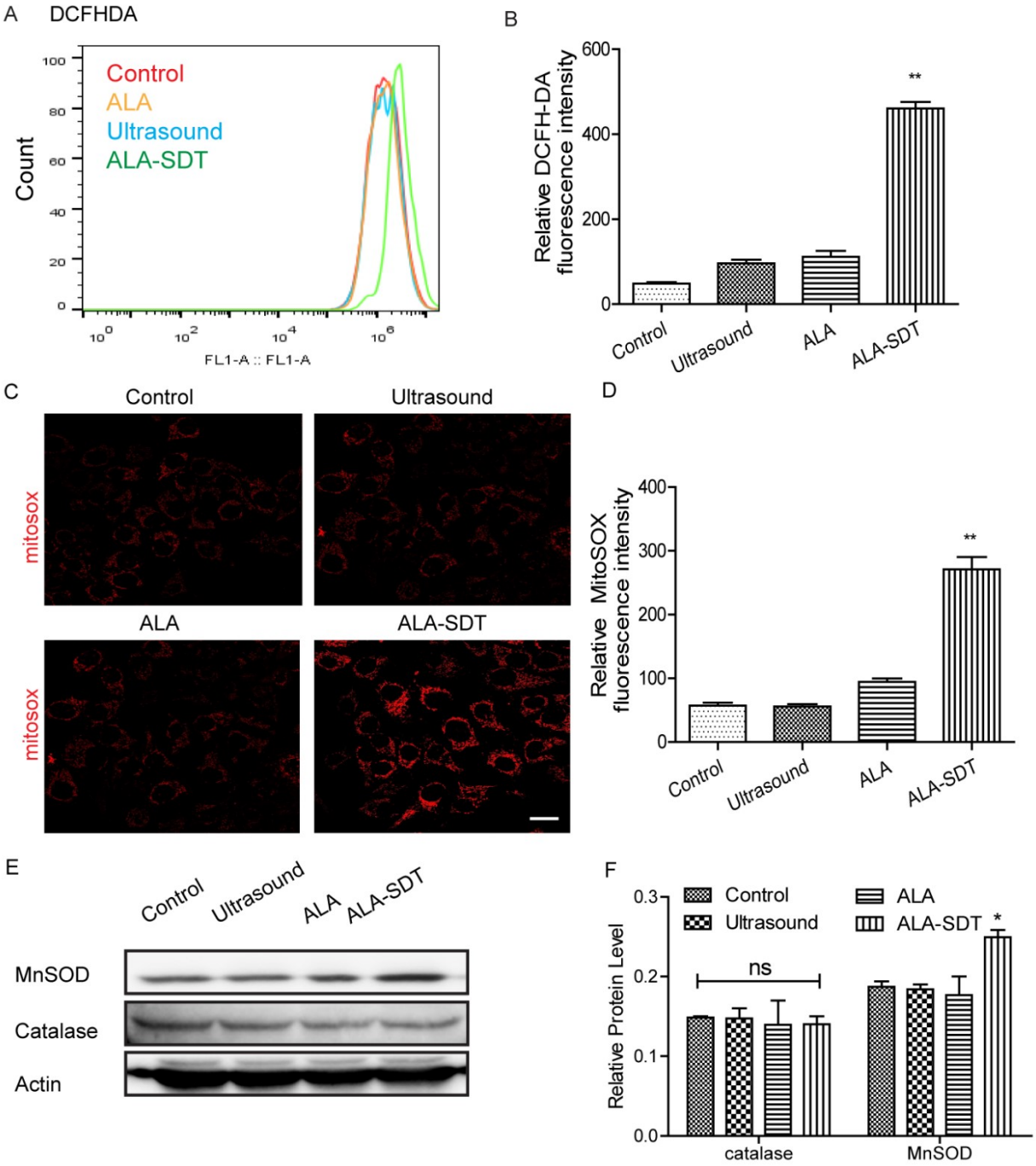
Figure 5. The PINK1-Parkin pathway can decrease the cell death and apoptosis in MCF-7cells induced by ALA-SDT (A, B) Applying Parkin siRNA to transfect MCF-7 cells 24h before ALA-SDT. Representative images showed the successful silencing of Parkin (A) with quantification data in (B). Quantitative analysis in three experiments, all these three experiments are independent of each other. *$p < 0.05$ vs. control. (C, D) ALA-SDT promotes mitophagy induced by in Parkin

siRNA transfected MCF-7 cells. (C) displays representative images and (D) displays quantification data. At least 25 cells were analyzed at each experiment. Quantitative analysis comes from three separate experiments. *$p < 0.05$ vs. control. Scale bar represents 50μm. (E, F) cell viability and cell apoptosis were measured 12 hours after Parkin knockdown and ALA-SDT treatment. Data represents with the mean± SEM from three independent experiments. *$p < 0.05$ vs. control.

3.6 ROS favored mitochondrial damage and subsequent mitophagy.

We then explored upstream regulatory mechanisms that lead to mitochondrial dysfunction and mitophagy abnormalities. More and more evidences showed that oxidative stress leads to mitophagy. Through flow cytometry, whether intracellular ROS and mitochondrial ROS increasing were tested after the treatment of ALA-SDT. It can be seen from Figures 6A and 6B that total intracellular ROS in MCF-7 cells was significantly increased 12 hours after ALA-SDT treatment compared to the control group. In the detection of mitochondrial ROS (Figures 6C and 6D, $p < 0.05$, $N = 3$) based on mitoSOX, we could observe similar trends. We explored the expression of MnSOD and catalase with Western blot after ALA-SDT treatment. MnSOD level increased while almost no change of catalase was detected (Figures 6E and 6F, $p < 0.05$, $N = 3$). In order to confirm what role the ROS is in mitophagy in ALA-SDT, a ROS scavenger, N-acetyl-L-cysteine (NAC) was applied to treated MCF-7 cells. Pretreatment with NAC showed a significant blockade of ALA-SDT-induced decrease of mitochondrial membrane potential (Figure 6G, $p < 0.05$, $N = 3$). Consistent with this finding, ATP production of ALA-

SDT group was increased by NAC compared with control group (Figure 6H, $p < 0.05$, $N = 3$). In addition, NAC almost completely inhibited the occurrence of mitophagy. It can be seen from Figures 6I and 6J, NAC pre-treatment brought down the co-localization of mitochondria and lysosomes, indicating a lower incidence of mitophagy. These results suggest that the induction of ROS in favor of mitophagy occurs after ALA-SDT.



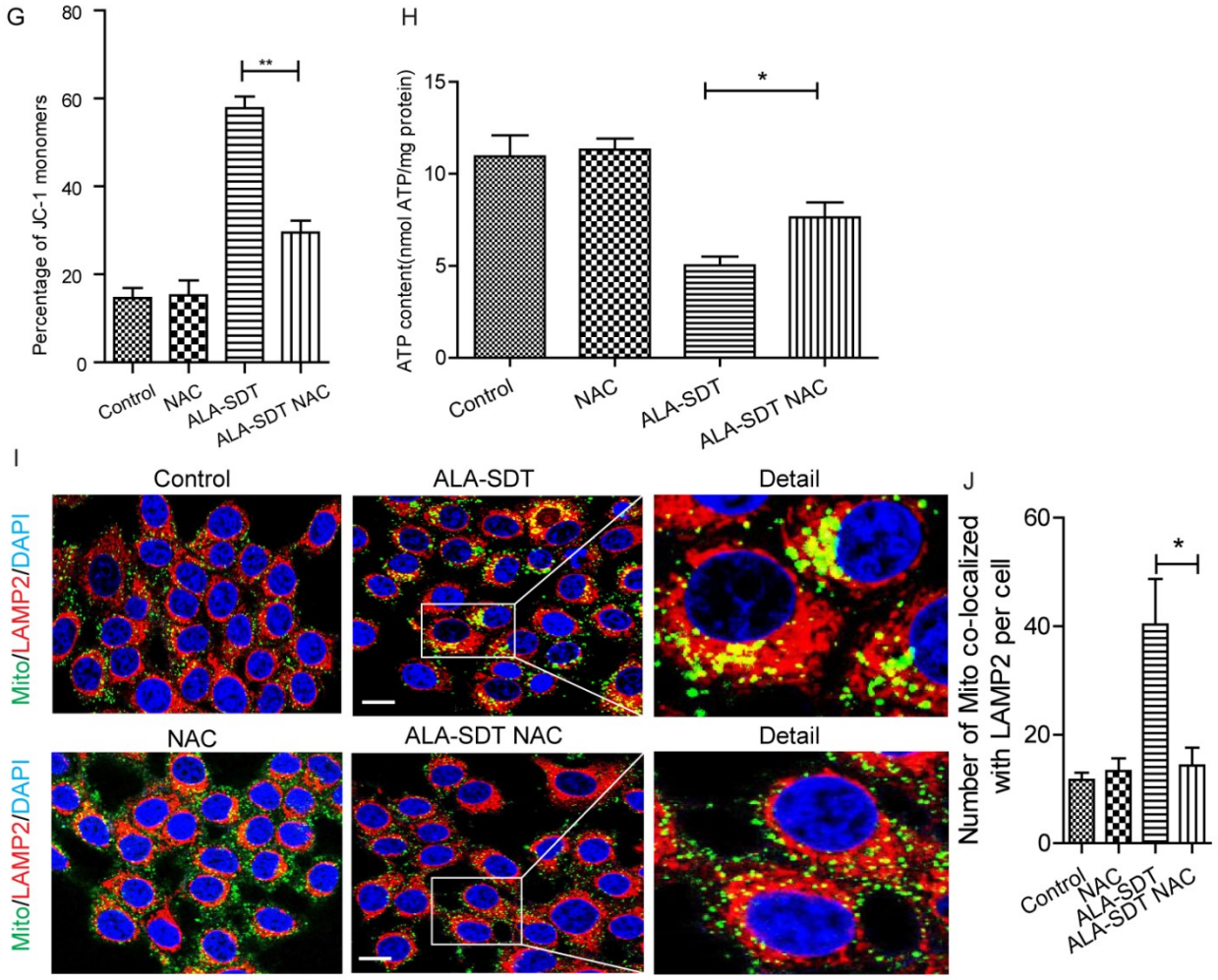


Figure 6. The buildup of ROS plays a crucial role in mitophagy induced by ALA-SDT. (A, B) After ALA-SDT, DCFH-DA was used to stain Intracellular ROS and the flow cytometry is applied to analyze it. The values with the mean ± SEM of three independent experiments. **$p < 0.01$ vs. control. (C, D) After ALA-SDT, mitoSOX was used to stain mitochondrial ROS and flow cytometry was applied to analyzed it. The mean ± SEM of three independent experiments are regarded as the values. **$p < 0.01$ vs. control. Scale bar represents 50μm. (E, F) Western blot assess the MnSOD and catalase levels. (E) shows representative images and (F) displays quantification data. The values with the mean ± SEM of three independent experiments. *$p < 0.05$ vs. control. (G, H) 1mM NAC was utilized to pretreated cells overnight and then the cells treated by the ALA-SDT. Mitochondrial membrane potential and ATP production were determined separately. The values with the mean ± SEM of three independent experiments. *$p < 0.05$ vs. control. **$p < 0.01$ vs. control. (I, J) It can be determined the occurrence of mitophagy. The representative image is displayed in (I) using the quantitative data in (J). The values with the mean ± SEM of three independent experiments. *$p < 0.05$ vs. control. Scale bar represents 50μm.

Chapter 4 Conclusion

Mitochondria plays a fundamental role in regulating cell function. Although cancer cells relatively have nothing to do with ATP-producing mitochondrial oxidation pathway, mitochondria are still decisive in regulating cellular homeostasis and cell death (Hsu, Tseng et al. 2016, Vyas, Zaganjor et al. 2016). Rapid morphological changes happen in mitochondrial fission and fusion as a response to physiological and pathological conditions (Chen and Chan 2005, Ussakli, Ebaee et al. 2013, Srinivasan, Guha et al. 2017). Mitochondrial kinetic dysfunction is associated with many neurodegenerative diseases, supporting the role of fission and nuclear fusion in maintaining cell homeostasis(Caino and Altieri 2016, Iommarini, Ghelli et al. 2017, Srinivasan, Guha et al. 2017). We observed that ALA-SDT can reduce mitochondrial membrane potential and ATP production, which means mitochondrial dysfunction has happened (Caino and Altieri 2016, Iommarini, Ghelli et al. 2017, Srinivasan, Guha et al. 2017). And the mitochondrial rupture caused by mitochondrial division is also triggered by ALA-SDT. We also found that the expression of mitochondrial fission protein FISF1 increased as the morphology of mitochondria changed from tubular, to punctiform, to mitochondrial fragmentation, while expression of mitochondrial fusion

protein MFN1 decreased. In conclusion, ALA-SDT induces mitochondrial dysfunction and leads to more fragmented mitochondria through fission.

As is known to all, mitophagy serves as a vital protective mechanism that selectively removes damaged mitochondria and maintains normal functional status of mitochondria to meet metabolic needs. Mitophagy can be triggered by dysfunctional mitochondria (Kubli and Gustafsson 2012, Kurihara, Kanki et al. 2012). The most complete documented signaling pathway controlling mitophagy is the PINK 1-Parkin pathway. PINK 1 or Parkin mutations cause mitochondrial dysfunction and are directly related with Parkinson's disease (de Vries and Przedborski 2013, Ryan, Hoek et al. 2015). In this study, the co-localization of mitochondria and autophagosome/lysosome was significantly increased; The expression of PINK1 was increased; Parkin was transferred from it. From cytoplasm to mitochondria, ALA-SDT induced mitochondrial autophagy in cultured MCF-7 cells and was engaged in the activation of the PINK 1-Parkin signaling pathway. Therefore, the PINK 1-Parkin pathway is a key to maintaining a healthy mitochondrial population by promoting mitophagy.

It has been reported that there exists an intricate crosstalk between mitophagy, apoptosis and cell death (Gump and Thorburn 2011, Zhang 2013). Normally, mitophagy should be protective in response to mitochondrial injury and damaged mitochondria. But in some cases, excessive mitophagy may leads to excessive degradation of the mitochondria, which ultimately leads to irreversible cell death - autophagic cell death (Kim, Rodriguez-Enriquez et al. 2007, Green and Levine 2014). Our study showed that Parkin knockdown lower cell viability and higher apoptosis rate, suggesting that ALA-SDT plays a protective role of mitophagy. This means that mitochondrial autophagy has an undiscovered function in regulating the sensitivity of SDT to kill the tumor. And drugs designed for mitochondrial autophagy can promote ALA-SDT to be more efficient.

Many studies have illustrated how ROS works in pathological conditions and how apoptosis was initiate by it (Ling, Liebes et al. 2003, Di Meo, Reed et al. 2016). ROS mainly target at mitochondria, which are considered to provide most of ROS within the cell (Mao, Yu et al. 2008, Schieber and Chandel 2014). We show that ALA-SDT induced significant accumulation in cellular and mitochondrial ROS, and increased expression of cellular proteins and mitochondrial antioxidant. Antioxidant imbalance - which means that oxidation will occur after ALA-SDT. ROS has been shown

to cause activation of multiple signaling pathways, mitochondrial dysfunction, and induction of mitophagy. In this study, the ROS inhibitor NAC was used to reduce intracellular and mitochondrial pro-apoptotic functions and explore its role in apoptosis of ALA-SDT cells. NAC significantly inhibited ALA-SDT induced mitochondrial membrane potential and ATP production. At the same time, ALA-SDT-induced mitophagy decreased significantly after the NAC treatment. These results indicate that ALA-SDT-induced ROS formation is an important intracellular factor that triggers mitophagy. In this article, we demonstrate that ALA-SDT mediates the production of excess ROS that can make mitochondrial dysfunction and lead to mitophagy. We have also found, PINK 1-Parkin signal pathway involved in ALA-SDT mediated mitophagy protects the cell viability and mitochondrial function under oxidative stress.

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