Winning the Nobel Prize Accelerates

Clinical Applications of Autophagy

Autophagy is not simply a recycling process involving the degradation of proteins and other cellular components. Research is also progressing on various disease-related fronts. Expectations centering on potential clinical applications rose when Yoshinori Ohsumi, the Tokyo Institute of Technology Honorary Professor often called 'the father of autophagy,' was awarded the 2016 Nobel Prize in Physiology or Medicine. In this feature, Professor Noboru Mizushima of the University of Tokyo, who has contributed to development of interdisciplinary research into autophagy, joined TMDU researchers involved in autophagy research to discuss the current status of autophagy research and future prospects.

"Elucidating a novel mechanism of autophagy for application to disease pathology"

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"Elucidating the mechanism and functions of autophagy"

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"Finding autophagy-based therapies for treating inflammatory bowel disease"

Professor
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"Finding the role of autophagy in cancer by genomic analysis"

Professor
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"Elucidating the role of autophagy in neurodegenerative disease"

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History of autophagy research

1955

Christian de Duve discovers lysosomes via the fractionation of rat liver cells. In 1956, he reports electron microscope evidence for lysosomes being cell organelles.

1963

de Duve coins the term "autophagy" to describe the mechanism of intracellular protein degradation.

Christian de Duve (1917-2013), who coined the term "autophagy"

1988

At the University of Tokyo, Yoshinori Ohsumi (now Honorary Professor, Tokyo Institute of Technology) is the first in the world to use an optical microscope to observe autophagy-related vesicles inside the vacuoles of starved yeast cells.

1993

Budding yeast mutants defective in autophagy gene(*atg* mutans) are isolated, leading to an explosion in autophagy research.

1997

Ohsumi identifies the autophagy-related gene *ATG1*.

2004

At the National Institute for Basic Biology, Noboru Mizushima (now Professor, The University of Tokyo) creates transgenic mice to enable fluorescent labeling of autophagosomes.

2004

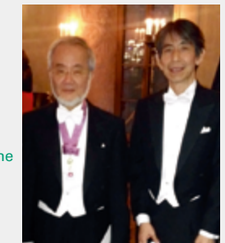
Mizushima creates ATG5 knockout mice lacking this specific autophagy gene.

2011

Mizushima creates ATG knockout mice lacking specific autophagy genes to enable mosaic analysis of autophagic function in all body organs.

2016

The Nobel Prize in Physiology or Medicine is awarded to Hon. Prof. Yoshinori Ohsumi.



Profs. Ohsumi and Mizushima at the Nobel Prize ceremony, Stockholm

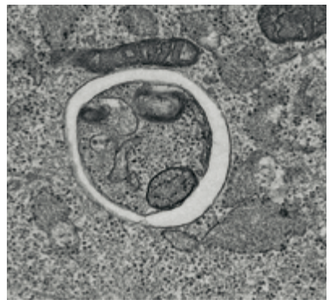
Autophagy Researchers Discuss Their Field

Progress and prospects in disease elucidation, drug discovery and therapy development

Current status of autophagy research

Shimizu: First, I would like to ask Prof. Mizushima how he felt when Prof. Ohsumi, with whom he conducted research into autophagy for many years, won the Nobel Prize.

Mizushima: I did not think that our work would be awarded such a prize because the work on autophagy has not yet reached the level of developing practical applications that benefit society. Winning a Nobel Prize is recognition of the basic science, and so is extremely gratifying for me as a researcher focused on the fundamentals. At the same time, I think it puts pressure on us to derive something useful from the field.



Mouse embryonic fibroblast cells in starvation (photo by Yuriko Sakamaki, TMDU)

Shimizu: Autophagy has certainly already had a substantial impact in medicine and biology. When I began research into cell death to find solutions to problems encountered with liver transplantation, I found autophagy was one of our main research topics. Can I ask what led everyone else here to engage in research into autophagy?

Okazawa: In my specialist field of neurodegenerative disease, we are looking for evidence of various cellular phenotypes, which are not necessarily related to autophagy. It is hard to observe changes inside the human brain, but we have been able to use dual-photon microscopes to see changes in neuronal autophagosomes inside the brains of living mice. Our hope is to be able to do something similar with human subjects.

Inazawa: For me, the link between autophagy and cancer was highlighted by a paper demonstrating the lower malignancy of mammary epithelial tumors in beclin-1 heterozygous knockout mice. Our research into the link between autophagy and tumorigenesis has shown that autophagy can promote cell survival in advanced cancer, making it something of a double-edged sword in this regard.

There are numerous clinical trials underway in the US of autophagy inhibitors in combination with existing anticancers drugs, and we are looking at autophagy research from this angle as well.

Watanabe: A human genome-wide association study (GWAS) published in 2007 identified genes that are critical to autophagy as potential causes of inflammatory bowel disease (IBD). This turned the attention of IBD researchers to autophagy as a new topic to study. One of these genes was ATG16L1, originally discovered by Prof. Mizushima. Naturally we were delighted that he teamed up with us here at TMDU. Unfortunately, since ATG16L1 mutations are uncommon among Japanese, we have proposed using knockout mice, antibodies and other analytical tools to study this area with him.

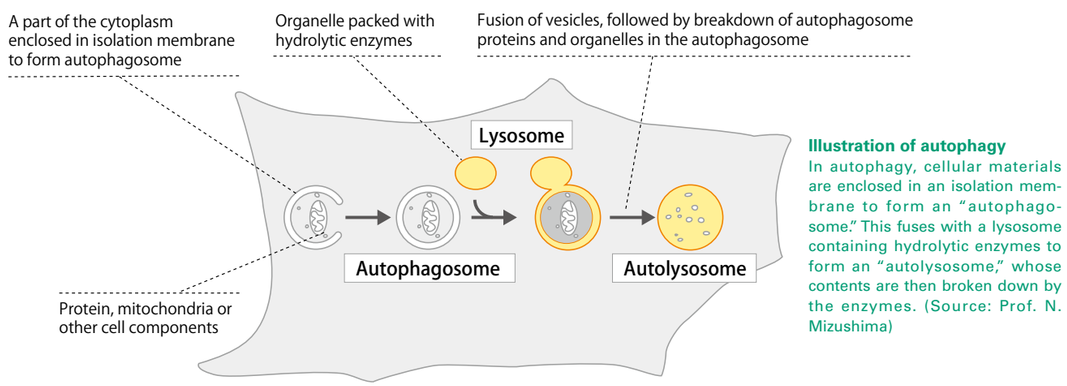
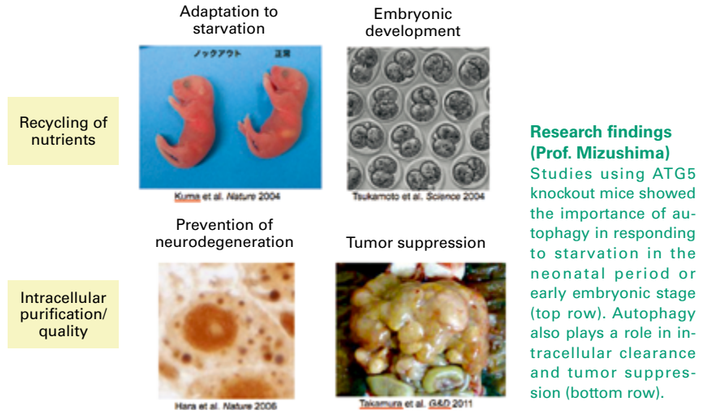


Illustration of autophagy
In autophagy, cellular materials are enclosed in an isolation membrane to form an "autophagosome." This fuses with a lysosome containing hydrolytic enzymes to form an "autolysosome," whose contents are then broken down by the enzymes. (Source: Prof. N. Mizushima)



Research findings (Prof. Mizushima)
Studies using ATG5 knockout mice showed the importance of autophagy in responding to starvation in the neonatal period or early embryonic stage (top row). Autophagy also plays a role in intracellular clearance and tumor suppression (bottom row).

Shimizu: It almost goes without saying why Prof. Mizushima began research on autophagy, but autophagy research has changed significantly in the years since he began working in the area with Prof. Ohsumi. As I am sure Prof. Watanabe will agree, Prof. Mizushima's creation of knockout mice strains has substantially helped to broaden the scope of subsequent research.

Mizushima: The number of research papers on autophagy increased markedly from around 2004, which was the year when we were able to observe autophagy and its functional inhibition in mice. The number of researchers in the area has since exploded, although it remains difficult to monitor autophagy in a quantitative manner. There remain many issues, but I believe they will be resolved.

Shimizu: Prof. Ohsumi remarked in an interview that a great deal of research remains to be done on yeast.

Mizushima: I agree. The Nobel Prize was awarded for Prof. Ohsumi's discoveries of autophagy mechanisms, rather than their elucidation. We have found the fundamental factors, but we still do not know how all of them work together in autophagy or understand the mechanism of some parts of the process such as autophagosome closure. I think we have now reached the stage where we understand the important issues that we need to resolve.

Shimizu: We have achieved results on several autophagy research projects at TMDU. How far has the basic science progressed in this area?

Inazawa: Looking at The Cancer Genome Atlas (TCGA) database of over 4,000 oncogene sequences, we can identify missense or functional loss mutations in about 15% of the autophagy-related genes. In addition, we know cancer cells can contain loss-of-function mutations in autophagy-related genes such as ATG5. Our team is focusing on the functional characterization of each of those mutations.

Okazawa: Accumulation of altered proteins in the brain is a key characteristic of neurodegenerative conditions, and therefore the mechanism for elimination of abnormal proteins is equally important as the mechanism of deposition. It is now widely recognized that autophagy is a crucial part of this puzzle. The problem is that its role is complex, as evidenced in part by the significant differences between disorders.

For example, in Parkinson's disease there is evidence of a causal link to genetic mutations in two directly autophagy-related mitochondrial proteins called Parkin and PINK1, and in Alzheimer's disease the data suggest autophagy may

Professor
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[Major areas of research]

- **Elucidation of the mechanism of autophagy**
  Based on analysis of autophagosome formation factors and nutrition signaling induction factors, this work examined autophagy induced by starvation or fertilization in the neonatal or preimplantation periods and explained the physiological significance of constant, low-level autophagy.

- **Development of methods for monitoring autophagy**
  Involving the creation of animal models for detecting autophagy, including transgenic mice to enable the fluorescent labeling of autophagosomes and ATG5 knockout mice, this work developed standard methodologies for autophagy measurement and diagnostics.

- **Link between autophagy and disease**
  Mutations in the mutant autophagy-related gene WDR45 were identified as one of the causative factors in the neurodegenerative disease SENDA.

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[Major areas of research]

•Novel mechanism of autophagy

Discovery of "alternative macroautohagy": analysis of DNA damage-treated ATG5 knock-out cells revealed a new autophagy mechanism that did not rely on ATG5 or ATG7, genes previously thought essential for autophagic processes in mammals.

•Analysis of novel autophagy mechanism

Studies showed the new autophagy mechanism to be associated with a protein known as ULK1, and to operate in erythrocytes from which mitochondria have been removed. This work also showed that autophagy plays a role in protecting cell DNA from damage due to radiation or chemical exposure.

promote amyloid production. In addition, while we see intracellular aggregation of altered proteins in most degenerative conditions related to autophagy, proteins can accumulate both inside and outside cells in the case of Alzheimer's disease. This is not yet well understood.

Shimizu: What is happening in terms of clinical research?

Watanabe: Anti-TNF antibodies have proven highly effective in treating Crohn's disease, and have even been dubbed a 'miracle cure' for the condition. We have focused our research on the ubiquitin regulatory genes whose expression is turned on by TNF-alpha. Associate Prof. Oshima and his team have demonstrated the involvement of these genes in Crohn's disease, along with the role played by autophagy via ubiquitin. There is no doubt of the link between autophagy and IBD, but we still do not sufficiently understand its precise role.

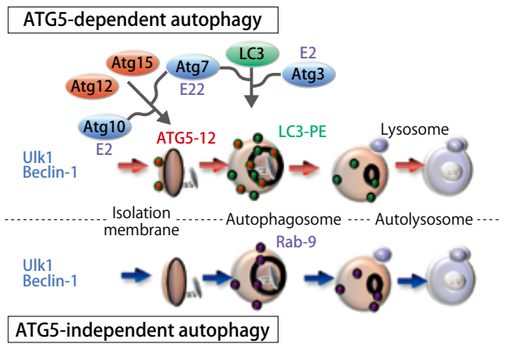
Autophagy and disease

Shimizu: We know that various diseases can arise as the functions of autophagy decrease. Conditions that have been clearly linked to autophagy genes include IBD and neurodegenerative disorders such as SENDA syndrome and Parkinson's disease. The regulation of autophagy genes is also believed to be a factor in polyglutamine diseases involving protein aggregation, suggesting possible treatments.

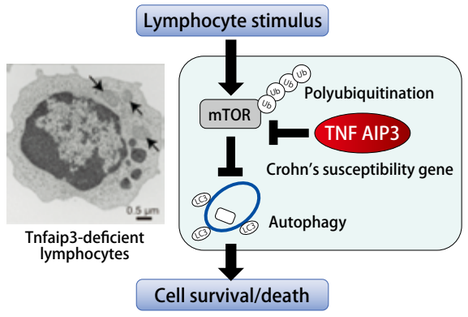
Watanabe: Environmental factors play a major role in Crohn's disease, and we think Paneth cells in the intestinal epithelium have a critical barrier function. Paneth cells tend to die in Crohn's patients. Students at our graduate school led by Associate Prof. Oshima have identified a possible new autophagy-related treatment approach based on blocking the signals that trigger the death of such cells. Westernized diets could also be an environmental factor, and we have found a new mechanism for induction of autophagy by fatty acids. Since the Paneth cells influence stem cell differentiation, we hope to build on these discoveries to find ways of restricting autophagy in the gut epithelium to restore the barrier function or else promote the functional recovery of stem cells.

Mizushima: While I think that autophagy is likely to be involved in these diseases, it does not mean that it is the cause. Autophagy genes in yeast are expressed almost entirely in autophagy alone, but in humans such genes are likely to have other functions besides autophagy.

Watanabe: You are correct. The enteritis that we study in mice will inevitably be different than that in humans. However, while it is hard to pinpoint the precise role of autophagy in disease, I think that demonstrating the existence



Research findings (Prof. Shimizu)
While traditional autophagy is ATG5-dependent, the new ATG5-independent mechanism known as alternative macroautophagy involves proteins such as ULK1, PI(3) kinases and Rab9.



Research findings (Prof. Watanabe)
Mitochondrial swelling and increased production of reactive oxygen species (ROS) are observed in Tnfaip3-deficient lymphocytes (photo). Signal pathway analysis demonstrated autophagy regulation by TNFAIP3 via polyubiquitination of the MTOR complex.

of autophagy-related mechanisms in clinical disorders could prove a valuable first step.

In this context, whether one adopts a fundamental or a clinical perspective can also affect things significantly. Naturally, we try to use both viewpoints in our research. Our policy is not to conduct any research unless it is useful to society.

Okazawa: Our objective is also to shed light on disease mechanism. This is slightly off topic, but in neurodegenerative disorders one of the problems is to identify at what stage the pathological trigger is activated. If this is before the stage of protein deposition, then we could not hope to prevent the pathological changes by activating autophagy pathways to eliminate aggregation.

Mizushima: But what about trying to activate autophagy mechanisms much earlier on? In the case of inheritable degenerative conditions, lowering the overall concentration of pathogenic proteins within the body might help to slow the progress of the disease.

Okazawa: That theory is most advanced in the case of Alzheimer's disease, where the idea would be to start treatment even in symptom-free patients as soon as a PET scan detected amyloid plaques.

Shimizu: Many researchers have now reported a link between Parkin and mitophagy (mitochondrial autophagy) in Parkinson's disease.

Mizushima: There is no doubt that Parkin is a causative factor, but there is not enough evidence to say it is indeed through autophagic insufficiency. Parkin is associated with mitochondrial degradation, but it also has many other functions, and we do not understand which types of functional loss lead to the development of Parkinson's disease.

Rather than such conditions, my view is that autophagy-based treatments could be more effective in diseases where autophagy is not compromised. If autophagy is not functioning properly, activating the system is unlikely to be effective, whereas promoting functionally active autophagy mechanisms could be useful in fighting disease.

Shimizu: In other words, we should view autophagy as a possible therapeutic target rather than as the cause of disease pathology?

Mizushima: Yes, I think that could more often be the case.

Shimizu: What about cancer? Various links to autophagy have been suggested in research to date.

Inazawa: That's right. However, we doubt that autophagy is playing a similar genetic role in cancer to the kinds of strongly tumorigenic 'driver' mutations associated with tyrosine kinases.

On the assumption that autophagy can either cause cancer or enhance tumor malignancy, we are studying the actual impact of autophagic pathway activation. Our view is that the therapeutic strategy of autophagy inhibitors in cancer could be the right clinical approach in a cellular context-dependent manner.

Mizushima: The difficulty in trying to utilize autophagy for therapeutic pur-

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[Major areas of research]

•Showing a common genetic link between autophagy and IBD pathology

Besides demonstrating ubiquitin-mediated regulation of autophagy by TNFAIP3, a gene strongly associated with inflammatory bowel disorders such as Crohn's disease and ulcerative colitis, this work revealed new mechanisms for autophagic involvement in apoptotic signaling and fatty acid induction of autophagy. Research continues into ubiquitin-mediated regulation of autophagy.

•Research using cultured human intestinal epithelial cells

Autophagy is being studied using epithelial cells cultured from patient biopsy specimens with the aim of finding autophagy-based therapies for treating IBD.

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[Major areas of research]

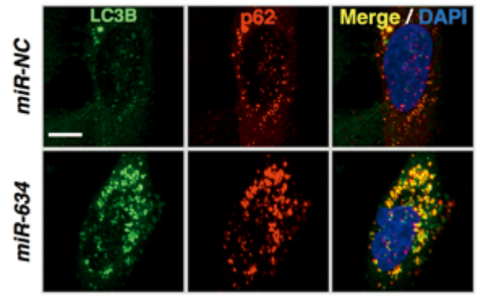
•Elucidation of the role of autophagy in cancer

This work aims to find new diagnostics and therapies for cancer by studying the links between autophagy and cancer stem cells, abnormal epithelial-mesenchymal transition (EMT) regulation in cancer, and tumor metastasis. It has demonstrated an autophagic connection in the pediatric cancer acute lymphoblastic leukemia (ALL), and has also shown a strong association between cancer pathology and inhibition of lysosomal degradation.

•Establishing new cancer diagnostics and treatments

The suppression of autophagy led to excessive ROS production inside cells. microRNA-634 was identified as a potential nucleic acid anticancer due to its ability to induce tumor cell death.

***MiR-634*-mediated autophagy inhibition is involved in enhancement of chemotherapy-induced cytotoxicity in ESCC cells**



Research findings (Prof. Inazawa)
Inhibition of autophagy via the expression of microRNA-634 results in the accumulation of large numbers of autophagosomes inside esophageal cancer cells (bottom row), as evidenced by staining of the autophagosome marker protein LC3B (green), the selective autophagy substrate molecule p62 (red), and a combination of the two (yellow).

poses is that it is not molecular in nature, but rather a functional process based on molecule aggregation. Drug discovery requires molecular targets to develop therapies, and autophagy simply does not fit the bill.

Proteasome inhibitors are another example of a type of cancer therapy—in this case, treatment of multiple myeloma—where the idea is to inhibit normal performance (of proteasomes) rather than taking advantage of abnormalities. There is a chance that autophagy could work in a similar way.

Challenges for clinical applications

Shimizu: Next, I would like to discuss autophagy-related drug development and clinical applications.

Inazawa: We are looking to use autophagic pathways for drug creation, partly due to the limitations of molecular target inhibitors and partly because we see a need for smart combination therapies with existing drugs to yield economically productive treatments. Our approach treats autophagic flux as the target.

Shimizu: In our laboratory, we have begun researching autophagy as an anticancer tool. We see it as one of the many possible therapeutic strategies that will be needed as personalized medicine advances.

Mizushima: One of the very convenient features of autophagy is that it functions regardless of the cellular contents being recycled. In the case of neurodegenerative disorders, it is thought that replacing the contents of cells to lower the concentration of toxic substances could delay the onset or progress of disease. There are many drugs available to treat cancer, but in contrast there are still virtually no effective therapies for treating neurodegenerative disorders. Autophagy could thus have a major therapeutic impact. The problem is that the time needed to show results implies extremely lengthy clinical trials, and it is not clear how this hurdle might be overcome.

Okazawa: Whilst the pathology of Alzheimer's can vary by patient and by the nature of symptoms, I think there is no doubt that autophagy could be effective in many cases. I would like to see us take advantage of it to help reactivate functionally impaired parts of autophagy flux, which should have clinical benefit. Yet the sheer length of the clinical trials that would be required is a major problem compared with, say, cancer. As Prof. Mizushima points out, the lack of precise biomarkers would also make it extremely difficult to detect significant differences in clinical outcomes in large-scale trials.

Shimizu: Looking ahead, the field of autophagy is expected to enter the clinical domain. Overseas, in some cases fundamental and clinical researchers are collaborating.

Mizushima: Even if this collaboration relies on a virtual set-up, it is a significant development if it enables collaboration between fundamental and clinical researchers.

Watanabe: Prof. Mizushima is also providing our team with the tools we need for research, but the opportunities for joint research with Prof. Shimizu or Prof. Inazawa are few and far between. As Vice-President of Research at TMDU, it pains me that we have not created more collaborative links between these centers of research and clinical excellence. We must generate opportunities to forge closer links between fields because autophagy is a vital phenomenon that could play a valuable role in various areas from basic science to clinical development.

Okazawa: Integrating the basic science with the clinical side is also an absolutely important part of our research in the field of neuroscience. While I am delighted that we understand the mechanism of autophagy, as a medical school graduate my goal is to apply the science to help people.

Watanabe: We have developed the technology to cultivate human intestinal epithelial cells. We want to use this technology to study autophagic variation and apply our understanding of the autophagy phenomenon in fundamental research.

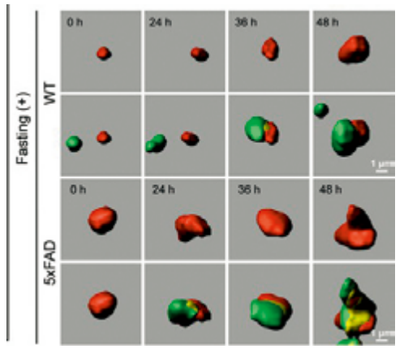
Shimizu: It would seem the primary issue is how to diagnose autophagy abnormalities.

Mizushima: With autophagy, it is not enough just to see the autophagosome, as we must also confirm that the contents of the autophagosome have been broken down. New detection methods have recently been developed to allow us to measure the autophagic degradation, and I believe this will make it easier to conduct autophagy research in mice. In humans, which are of far more interest, at present the only lead we have is to investigate abnormalities in autophagy due to related genetic mutations. We still have much to do to develop autophagic diagnostic and quantification tools.

Okazawa: PET scan technology enables us to observe a wide range of cellular events in neurons. Besides observing simple aggregates, we can also visualize the dynamics of various receptors. Observing the whole scheme of subcellular dynamics might not be easy yet, but I am confident the technology will develop further.

Inazawa: Autophagy diagnostics such as measurement of autophagy flux in cancer cells and/or tumor tissues will be an important part of developing cancer treatments based on the personalized or precision medicine approach. I am glad we had this opportunity for a discussion between researchers from different parts of TMDU.

Shimizu: Agreed. Let's try to help one another in advancing our autophagy research.



Source: http://www.nature.com/articles/srep12115

Research findings (Prof. Okazawa)
Neuronal cells from wild type (WT) or Alzheimer's model (5xFAD) fasting mice are injected either with TAMRA-beta-amyloid (top row) or TAMRA-beta-amyloid plus EGFP-Lc3 plasmid (bottom row) and then observed 24, 36 and 48 hours later to visualize the interaction over time between the endosomes (red) and autophagosomes (green).

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[Major areas of research]

•Development of technology to observe in vivo autophagy in brain neurons

Using fluorescent-labeled proteins, a method was developed to use dual-photon microscopes to see changes in neuronal autophagosomes inside the brains of living mice. These studies demonstrated the existence of starvation-induced autophagy in brain cells.

•Role of autophagy in Alzheimer's disease

This work shows that starvation-induced autophagy can potentially aggravate Alzheimer's disease by promoting beta-amyloid deposition inside brain cells.