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Short communication

Estimation of autophagy pathway genes for autophagy induction: Overexpression of Atg9A does not induce autophagy in recombinant Chinese hamster ovary cells

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ABSTRACT

Autophagy, a self-eating process, has attracted attention as a new target for anti-cell death engineering of recombinant Chinese hamster ovary (rCHO) cells in order to improve culture performance. In an effort to obtain genetic targets for autophagy control, changes in the mRNA and protein expression of four core autophagy pathway genes (*Ulk1*, *Beclin-1*, *Atg7*, and *Atg9A*) have been investigated in serum-free suspension cultures of rCHO cell lines. Among the four target genes, *Atg9A* was the only gene showing decreased levels of mRNA and protein simultaneously when comparing the expression in the late period of the culture showing the maximum level of autophagy with that in the control time point (Day 4). In order to verify the potential of *Atg9A* as an autophagy induction target, Atg9A was overexpressed in rCHO cells using the Tet-Off System. However, Atg9A overexpression did not significantly influence the autophagy induction and culture longevity. Taken together, the results obtained here demonstrate that the downregulation of *Atg9A* is not the sole limiting factor for autophagy induction in serum-free suspension cultures of rCHO cells. This suggests that combinatorial regulation of the genes in the upstream autophagy pathway with *Atg9A* overexpression could be a promising approach for autophagy control.

1. Introduction

Chinese hamster ovary (CHO) cells, which are the most widely used mammalian hosts for the production of therapeutic proteins, undergo programmed cell death, especially apoptosis, upon exposure to stress during cultures [1]. In order to increase the overall production yield, many anti-apoptosis engineering strategies have been developed for CHO cells [1].

Recently, autophagy has received attention as a new target for anti-cell death engineering [2]. Autophagy is a global catabolic process through which intracellular components in a double-membrane vesicle, termed an autophagosome, are recycled via lysosomal degradation. In response to sublethal stresses such as nutrient deprivation, autophagy can supply cells with metabolites by degrading the cytoplasmic materials for cell survival. Beyond this basic role, autophagy is also important in cell death and survival [3]. Unlike apoptosis, it is not determined whether autophagy is responsible for cell death or survival; rather, it is suggested that an optimal level of autophagy may be desirable to remove damaged organelles and to provide turnover of nutrients, resulting in increases in cellular efficiency and productivity [2].

Until now, studies on autophagy in CHO cell cultures have revealed the presence and activation of autophagy in batch [4] and fed-batch cultures [5,6]. In these studies, modulation of the autophagy pathway has depended entirely on chemical autophagy inhibitors such as 3-methyl adenine (3-MA) [6], bafilomycin A1 [5,7] or chemical autophagy inducers such as rapamycin [5,7] due to the limited information related to the genetic regulation of autophagy pathways in CHO cells. In a recent study, autophagy induction by treatment with rapamycin delayed the apoptosis induction and viability drop in CHO cells, resulting in an increase in the maximum antibody production during a serum-free suspension batch culture [8]. This suggests that additional induction of autophagy is beneficial to prepare for nutrient depletion and byproduct accumulation. However, chemical autophagy inhibitors and inducers, which lack complete specificity for the autophagy pathway, affect a wide range of cellular responses and appear to only exert partial effects on autophagy [9]. Therefore, it is necessary to investigate appropriate genetic targets for enhancing the overall autophagy pathway specifically.

The process of conventional autophagy consists of the formation of the isolation membrane and the autophagosome, autophagosome–lysosomal fusion, lysosomal digestion, and transport of the resulting macromolecules into the cytosol. Among these processes, the autophagosome formation is referred to as the 'core' machinery of the autophagy pathway [10]. These core Atgs proteins are composed of four molecular components. First, the Atg1/unc-51-like kinase (ULK) complex is responsible for the induction of autophagy. The mammalian homologs of Atg1 are Ulk1 and Ulk2. However, Ulk1 could be the major form because

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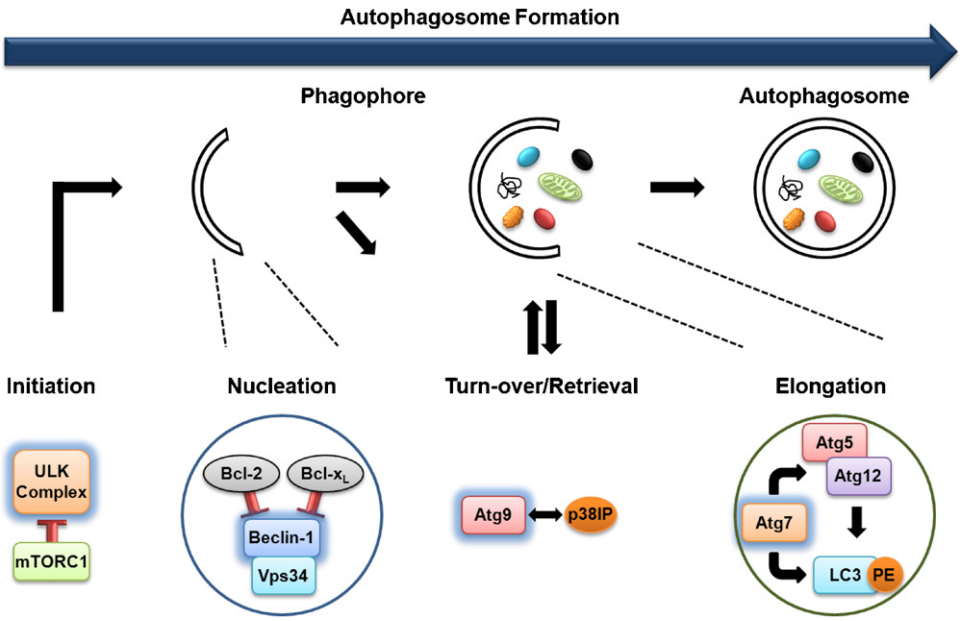

Fig. 1. Schematic depiction of the autophagosome formation process and its core molecular machinery in mammalian cells. For simplicity, only major autophagy pathway components are shown, and detailed information is described in Sections 1 and 3. The four autophagy pathway genes evaluated in this study are highlighted.

silencing Ulk1 alone is sufficient to inhibit autophagy in some mammalian cell lines including HEK293 cells [11]. Second, the class III phosphatidylinositol 3-kinase (PI3K)/Vps34 complex I is crucial in the initial stages of the vesicle nucleation/autophagosome formation. Beclin-1 regulates the lipid kinase Vps34 protein by interacting with several cofactors, and thereby inducing autophagy. In addition, Beclin-1 is a key molecule in the interaction between the autophagy and apoptosis by binding with the anti-apoptotic molecules, Bcl-2/Bcl-x_L. Third, two Atg7-mediated ubiquitin-like protein (Atg12 and Atg8/LC3) conjugation systems are required for the vesicle elongation and expansion, resulting in the autophagosome formation. This conjugation system is similar to a ubiquitination process, and the Atg7 proteins play the role of the E1-like enzyme. Fourth, Atg9 is speculated to be a carrier of lipids to form the autophagosomes. In mammalian cells, two functional orthologues of Atg9 (Atg9L1 and Atg9L2) exist, but Atg9L1, which corresponds to Atg9A, is only expressed ubiquitously [12].

In this study, the changes in the mRNA and protein expression levels of these four core autophagy-related genes (*ATG*), which are shown in Fig. 1, during serum-free suspension batch cultures of three different recombinant CHO (rCHO) cell lines were evaluated in an effort to search for appropriate target genes for pro-autophagy engineering. Furthermore, based on the mRNA and protein expression results, we attempted to modulate the autophagy pathway of an antibody producing rCHO cell line through the controlled overexpression of Atg9A, which only showed decreased expression of both mRNA and protein as the culture progressed.

2. Materials and methods

2.1. Cell lines and batch culture

Three different recombinant CHO (rCHO) cell lines producing erythropoietin (EPO) and antibodies (Ab1 and Ab2) were used in this study. The EPO [7] and Ab2 cell lines [13] were established previously in our laboratory; the Ab1 cell line was provided by ISU ABXIS (Seoul, Korea). All cell lines were adapted to grow in serum-free suspension culture in 125 mL Erlenmeyer flasks (Corning, Corning, NY) containing 50 mL of medium in a Climo-shaking CO_2 incubator (ISF1-X, Adolf Kuhner AG, Birsfelden, Switzerland) set at 110 rpm, 85% humidity, and 37 °C. SFM4CHO™ (Hyclone, Logan, UT) supplemented with 4 mM glutamine was used for the basal serum-free medium for the suspension culture. Different concentrations of methotrexate (MTX; Sigma–Aldrich, St. Louis, MO), 80 nM for EPO cell line, 300 nM for Ab1 cell line, and 1 μM for Ab2 cell line, were added to the serum-free medium, respectively. For the batch cultures, cells were inoculated at a concentration of 2.0×10^5 cells mL^{-1}. The viable cell concentrations were estimated using a hemacytometer, and viable cells were distinguished from dead cells using the trypan blue exclusion method.

2.2. Western blot analysis

Detailed information of the Western blot analysis has been described previously [4]. The antibodies used for analysis were anti-LC3 (Clone 51-11; Medical and Biological Laboratories, Nagoya, Japan), anti-Atg1/Ulk1, anti-β-actin (Clone AC-74; Sigma–Aldrich), anti-Beclin-1, anti-Atg7 (Cell Signaling Technology, Beverly, MA), and anti-Atg9A (Abcam, Cambridge, UK).

2.3. Quantitative real-time PCR (qRT-PCR) analyses

The total RNAs from each individual clone were isolated using a RiboEX column™ total RNA Purification kit (GeneAll, Korea) and the cDNAs were prepared using a Super-Script™ first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturers' protocols.

The primers for qRT-PCR were designed using Beacon Designer™ (PREMIER Biosoft International, Palo Alto, CA) and PrimerQuest℠ (Integrated DNA Technologies, Coralville, IA) against partial or full coding sequences for four *ATG* of the CHO cells, which were sequenced based on homology between mouse, rat, and human.

For each cDNA sample, the qRT-PCR was performed in triplicates using iQ™ SYBR® Green Supermix (Bio-rad) on a Bio-Rad CFX96 machine according to the manufacturer's protocols. Each PCR reaction included a reaction mix without a template in order to verify the potential reagent contamination. The relative changes in gene expression with respect to the control time point (Day 4) were calculated using a delta-delta threshold cycle ($\Delta\Delta C_T$) method [14].