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Autophagic response to cell culture stress in pluripotent stem cells

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ABSTRACT

Autophagy is an important conserved cellular process, both constitutively as a recycling pathway for long lived proteins and as an upregulated stress response. Recent findings suggest a fundamental role for autophagic processes in the maintenance of pluripotent stem cell function. In human embryonic stem cells (hESCS), autophagy was investigated by transfection of LC3-GFP to visualize autophagosomes and with an antibody to LC3B protein. The presence of the primary cilium (PC) in hESCs as the site of recruitment of autophagy-related proteins was also assessed. HESCs (mShef11) *in vitro* displayed basal autophagy which was upregulated in response to deprivation of culture medium replacement. Significantly higher levels of autophagy were exhibited on spontaneous differentiation of hESCs *in vitro*. The PC was confirmed to be present in hESCs and therefore may serve to coordinate autophagy function.

1. Introduction

Human embryonic stem cells (hESCs) *in vitro* are particularly sensitive to culture conditions and require very tight maintenance of their cellular homeostasis for cell maintenance and survival. Compared with many other cell lines, hESCs usually die when cell culture is disrupted even for a short period. For example, during conventional passage in 2D adherent culture from one vessel to another, less than 20% of cells may survive without the presence of apoptotic inhibitor especially when single cells [1]. This makes amplifying and scaling up hESCs inefficient and costly. An understanding of how common cellular stresses, whether external or internal (e.g. oxidative and endoplasmic reticulum stress), act on hESC homeostatic equilibrium and are subsequently transduced into a response is therefore important for developing effective methods of culturing these cells for regenerative medicine applications.

Apoptosis is the most widely studied mechanism related to cell death or survival in hESCs *in vitro* and various apoptotic related genes have been identified that shift the balance of cell survival in culture with chemicals (e.g. rho kinase inhibitor, pinacidil) used to prevent initiation of apoptotic pathways [1,2]. While apoptosis is a decisive way of dealing with cellular stress by bringing about the elimination of the cell in tissues or in culture, other cytoprotective mechanisms are equally important and aim instead at cellular self-preservation. The most common of these mechanisms is autophagy, or at its simplest, self-digestion. Attention has turned to this stress adaptation process in stem cells because as well as being a means of cytoprotection it may have an important role in stem cell self-renewal, differentiation and reprogramming.

During autophagy the cell encloses portions of its cytoplasm within a unique vesicle specifically targeted for degradation. Macrophagy is the most common pathway where the cytoplasmic contents are enveloped within a characteristic double-membraned autophagosome, which then fuses with a lysosome to form the autolysosome. The inner autophagosomal membrane breaks down after fusion and lysosomal hydrolases diffuse into the main body of the autolysosome to break down its contents [3]. Autophagy is a process that can occur on a basal level to allow cells to efficiently recycle long lived proteins but also is crucial in times of cellular stress for cell survival. For example, during nutrient deprivation or starvation, the cell is able to increase its autophagic degradation to meet its metabolic demands. As a result, autophagy can be seen to be a pro-survival mechanism rather than a cell death or elimination mechanism of apoptosis. As with all cellular processes however, autophagy must be kept in balance. Too much autophagy and cells will undergo autophagic cell death which can be distinguished from apoptosis.

Microtubule-associated protein 1 light chain 3, LC3, is often used as a marker for the autophagosome. In humans there are three

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isoforms; LC3A, LC3B and LC3C, although it is LC3B which is associated mostly with autophagy. The functions of LC3 within the autophagosome are not completely clear; however it is thought that LC3 acts to promote tethering and fusion of the autophagosomal membrane. In addition, LC3 may have a role in the selection of cargo to be loaded into the autophagosome [4].

As of yet, the process of autophagy within pluripotent stem cells whether human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPS) has not been fully explored. In other human stem cells, high levels of autophagy have been implicated in maintaining the characteristic of cell 'stemness' [5]. Conversely, autophagy has been shown to be upregulated during hESC differentiation [6]. The presence of the autophagosome in hESCs has been shown initially both through lentiviral transduction and subsequent visualisation of LC3-GFP [6] and through immunofluorescence using the LC3B antibody [7].

Recently the primary cilium (PC) has come under scrutiny as a crucial cell organelle to sense and monitor the extracellular milieu. Its role in the induction of autophagy was demonstrated during starvation of the cell which is dependent on a functional cilium and canonical hedgehog signalling pathways [8]. Previously, hESCs had been shown to exhibit a PC [9] and hence it is likely that cross-talk between autophagy and cilia function plays a fundamental role in cellular homeostasis and survival of hESCs. Here we further explore the role of autophagy within hESC cell lines *in vitro* by visualizing autophagosomes using transient transfection of LC3-GFP and an LC3B antibody to ascertain the degree autophagy upregulation in response to starvation and investigate the presence of the PC on pluripotent cells.

2. Materials and methods

2.1. Cell culture

HeLa cells were grown in uncoated T25 flasks in 10 ml of Fibroblast Growth Medium, FGM (50 ml Fetal bovine serum to 450 ml DMEM) (Life Technologies). The cells were passaged every 7 days using TrypLE select (Life Technologies) with a split ratio of 1:10 and were fed fresh FGM every 3 days. hESC cell lines mShef11 and mShef 13 (derived under license from the Human Fertilisation and Embryology Authority, HFEA) were cultured on CELLstart (Life Technologies) matrix with Nutristem (Biological Industries). Each flask was fed daily with 5 ml of medium and manually passaged with a split ratio of 1:3. For spontaneous differentiation Nutristem medium was replaced with 7 ml FGM, for a minimum of 7 days. Cells were passaged after reaching 90–100% confluency, using trypsin TrypLE select with a split ratio of 1:6.

2.2. Transient transfection of HeLa cells and hESCs

Competent cells (Invitrogen) were transformed with the pSelect-GFP-LC3 plasmid (InvivoGen) using a heat shock technique. 100 μl of competent cells were incubated on ice with 50 ng of the pSelect-GFP-LC3 circular DNA and placed in a water bath at 42 °C for 45 s and returned to ice for 2 min. Trypticase soy agar (TSA) antibiotic free broth was added and the mixture incubated for 1 h at 37 °C. Cell suspension was spread across LB plates with zeocin (LB solid media from InvivoGen) and colonies grown up overnight at 37 °C. 5 ml of liquid LB broth with zeocin (InvivoGen) was inoculated with a single colony and incubated overnight at 37 °C on a rocker. Once cultured, bacterial culture was centrifuged and the pellet refrigerated at 4 °C. DNA was prepared using a Plasmid Plus Midi Prep Kit (Qiagen) according to manufacturer instructions. 5 μg of GFP-LC3 plasmid DNA was diluted in 250 μl of OPTI-MEM (Life Technologies) and 12 μl of Lipofectamine2000 (Life Technologies) was diluted in another 250 μl of OPTI-MEM. The diluted DNA and diluted Lipofectamine2000 were then combined and incubated at room temperature for 30 min. The media was aspirated from the cells to be transfected and 2.5 ml of fresh media was added. Finally 500 μl of the DNA-Lipofectamine2000 solution was added to each well. The cells were incubated at 37 °C for 16 h with the transfection media.

2.3. Immunocytochemistry

Cell samples were fixed with methanol for 15 min at room temperature and then permealised using 0.2% Triton X containing FBS in PBS for 1 h. Blocking buffer was removed and fixed cells were incubated at 4 °C overnight with rabbit anti-LC3B antibody, 1:500 dilution; Cell Signaling Technology), washed 3 times with PBS and incubated with secondary antibody was applied at 4 °C for 1 h (anti-rabbit Alexa Fluor 488, 1:100 dilution; Life Technologies). Preparations were counterstained with Hoescht 33342 (1:10000 dilution in PBS) for 5 min at room temperature. The negative staining control was secondary antibody only. Alternatively to localise the PC in pluripotent hESCs fixed cells were incubated directly with anti-acetylated tubulin antibody and anti Oct4 conjugated antibody (Millipore).

2.4. Fluorescence microscopy

Transfected and stained cells were visualised using the AMG EVOS inverted digital microscope. Images were taken at ×20 and ×40 magnification using the following channel settings. GFP; intensity = 100% exposure = 60 ms, DAPI; intensity = 50% exposure = 30 ms.

2.5. Time lapse microscopy

Transfected cells were visualised and incubated in the Nikon Biostation CT with pictures taken at ×10, ×20 and ×40 magnification at 1 h intervals. Both transmitted light and green fluorescence images were taken.

3. Results

The clinical grade Master(m)Shef11 cell line and HeLa cells (transfection control) were transfected using a GFP-LC3 plasmid. After transfection, cell lines were subjected to flow cytometry and time lapse microscopy (Nikon Biostation) to quantify their transfection efficiency and cell proliferation. Transfection efficiency of HeLa cells was approximately 38% for GFP-LC3 while an efficiency of 14% was observed for mShef11 cells. The reduced rate of transfection for hESCs reflects the relatively poor transfection efficiency in these cells. There was no obvious difference in proliferation rate of transfected or non-transfected mShef11 cells in culture.

Autophagy is normally upregulated during nutrient deprivation [10]. For optimal maintenance of hESCs, culture medium is usually replaced completely every day. Hence, the effect of cell deprivation and starvation was investigated by not replacing medium after initial plating. This culture regime lengthened the period and intensity of LC3-GFP expression, with significantly more fluorescence seen at 24–36 h in deprived cells compared to the control cells (Fig. 1).

As another method to confirm autophagy, immunolocalisation of the autophagosome marker LC3B was undertaken in mShef11 cells displaying high degree of pluripotency or in cells that had undergone spontaneous differentiation for 7 days. LC3B was localised around the cell nuclei and distributed in the cytoplasm. A lack of many distinctive fluorescent puncta suggested only basal

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