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Autophagy in human skin fibroblasts: Comparison between young and aged cells and evaluation of its cellular rhythm and response to Ultraviolet A radiation

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Synopsis

Autophagic mechanisms play critical roles in cell maintenance. Damaged organelles that are not removed by autophagosomes, which act by engulfing and degrading these cellular components, have been linked to various pathologies. Recently, the progression of aging has also been correlated to a compromised autophagic response. Here, we report for the first time a significant reduction in autophagic levels in synchronized aged normal human skin fibroblasts as compared to young fibroblasts. We measured a 77.9% reduction in autophagy as determined by reverse transcription-polymerase chain reaction for LC3B expression, a microtubule-associated protein correlated to late stage autophagosome formation. In addition, we visualized these same changes by immunocytofluorescence with antibodies directed against LC3B. By harvesting synchronized, as well as unsynchronized cells over time, we were also able to measure for the first time a nighttime peak in autophagy that was present in young but absent in aged fibroblasts. Finally, since human skin is constantly subjected to environmentally induced oxidative stress from sunlight, we exposed fibroblasts to 10 J/cm^2 ultraviolet A and found, in good agreement with current literature, not only that irradiation could partially reactivate autophagy in the aged cells, but also that this increase was phase shifted earlier from its endogenous temporal pattern because of its loss of synchronization with circadian rhythm.

INTRODUCTION

Autophagy, a major cellular degradative and recycling pathway, has now been shown to be essential for health and longevity, as well as a critical player in the aging process (1). As a highly conserved mechanism, it is responsible for the continuous recycling and renewal of intracellular organelles, lipids, and proteins and is a vital secondary source of energy for cells. It is a well-calibrated pathway that supports cellular homeostasis and responds to stress. By ensuring that malfunctioning or damaged intracellular components are removed and recycled, further intracellular damage can be minimized. The autophagic process removes cellular debris by first sequestering material in an autophagosome, followed by

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fusion with a lysosome leading to the formation of an autolysosome, which then degrades and recycles cellular components. Autophagy can be considered to be a very efficient quality control mechanism that maintains cell efficiency and health (2).

Decreases in autophagic activity have recently been associated with age. For example, hepatocytes were found to accumulate intracellular damage over time (3) and the loss of activity as described by Cuervo (4) has now been recognized as a major aging pathway. Furthermore, the link between impaired autophagy and age-associated illness has now been established for cancer, diabetes, and Alzheimer's and Parkinson's diseases and is currently an active area of investigation. Aging tissues show an expansion of lysosomal compartments, accumulation of autophagic vacuoles, and deposition of undigested materials inside the cells. These changes result in a decrease of energy supply and an increase in intracellular damages and oxidative stress, all of which have been shown to accelerate aging.

Since metabolic intermediates are generated as a consequence of autophagy, caloric restriction has been shown to play a role in the life span extension associated with autophagy and this has been genetically confirmed in *Caenorhabditis elegans* (5). In addition, circadian rhythms, which also coordinate metabolic activity, have been observed in yeast to temporally affect autophagy (6). To understand how these processes may influence cutaneous health and aging, we compared autophagy levels by measuring LC3B, a microtubule-associated protein correlated to late stage autophagosome formation (7) between young and aged skin fibroblasts, and then also analyzed these levels as a function of time. Furthermore, since human skin is constantly exposed to sunlight, we determined their response to environmental stress induced by ultraviolet A (UVA; 320–400 nm).

MATERIALS AND METHODS

TISSUE CULTURE

Normal human dermal skin fibroblasts from 2 day and 67-year-old donors were obtained from The Coriell Institute (Camden, NJ) and grown on 4-well chamber slides (2×10^4 cells/well) for immunocytofluorescence or on 60-mm dishes for RNA extraction (5×10^4 cells/dish). They were cultured in Dulbecco's Modified Eagle's Media (DMEM) media with 5% bovine calf serum and 1% penicillin/streptomycin at 37°C in a 5% CO_2 humidified incubator. Cells were cultured for 72 h before the start of the experiment.

CELL SYNCHRONIZATION

Cells were synchronized via serum starvation. Cells to be synchronized were washed once with Dulbecco's Phosphate Buffered Saline (D-PBS) and then treated with medium without serum. Medium with serum was placed on cells that were not synchronized. Cells were incubated for 24 h. After synchronization, the media were removed and medium with serum was added to both unsynchronized and synchronized cells. RNA was extracted at 0, 1, 2, 4, and 8 h after release from starvation. In a second experiment, synchronized cells were irradiated just before release from starvation and RNA extracted at the same time intervals as before.

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Cells were fixed with 4% paraformaldehyde and then permeabilized with methanol. Cells were blocked with 5% goat serum and 0.3% Triton X-100 (Sigma-Aldrich, St. Louis,

MO) before being probed for LC3B with a rabbit polyclonal antibody, which the manufacturer stated to have minimal cross-reactivity (Cell Signaling Technology, Danvers, MA) at a 1:100 dilution in block solution overnight at 4°C. Then the cells were washed and incubated with an Alexa Fluor® 488 F(ab') 2 Fragment of Goat Anti-Rabbit IgG (H+L) antibody (Invitrogen, Carlsbad, CA) at 1:400 in block solution for 1 h at room temperature in the dark. Cells were washed and then the chambers were removed from the slides. Slides were mounted using Vectashield with 4',6-diamido-2-phenylindole (DAPI) (Vector, Burlingame, CA). Cells were visualized with a fluorescence microscope using an FITC filter.

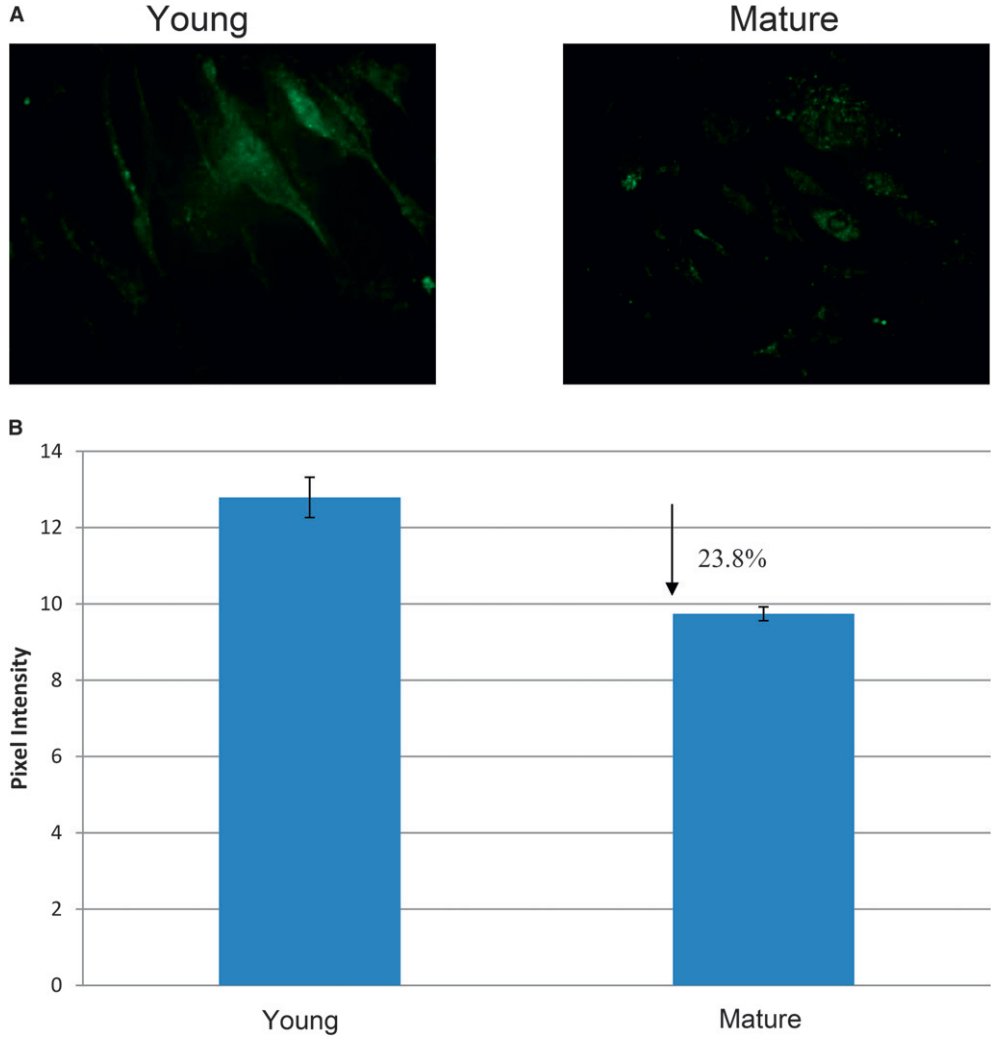

Figure 1. (A) Immunocytofluorescence of young and aged fibroblasts comparing LC3B levels show a reduction in autophagy in the mature cells (magnification = 20×). (B) Graph summarizing pixel intensity from immunocytofluorescent microscopic images of young and aged fibroblasts (n = 4). Autophagy decreased 23.8% (±1.9% SEM).

RNA ANALYSIS

RNA was extracted with the RNeasy Kit (Qiagen, Valencia, CA) and prepared as directed in the manufacturer's instructions. RNA concentration was determined by UV absorbance at 260 nm. For reverse transcription-polymerase chain reaction (RT-PCR), RNA was reverse transcribed using a High-Capacity cDNA Archive Kit (Applied Biosystems, ABI, Foster City, CA), as per the manufacturer's instructions. Real-time PCR was employed using the 2X TaqMan Fast Universal PCR Master Mix (ABI) in conjunction with a 20X TaqMan Gene Expression Assay Mix, which consists of human primers and probes for MAP1LC3 (ABI, cat# Hs00797944_s1) and the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ABI cat# Hs99999905_m1). Relative gene expression [change in expression of target gene normalized to an endogenous control (GAPDH) and relative to a reference group (untreated cells)] of real-time RT-PCR data was calculated using the $2^{-\Delta\Delta CT}$ method.

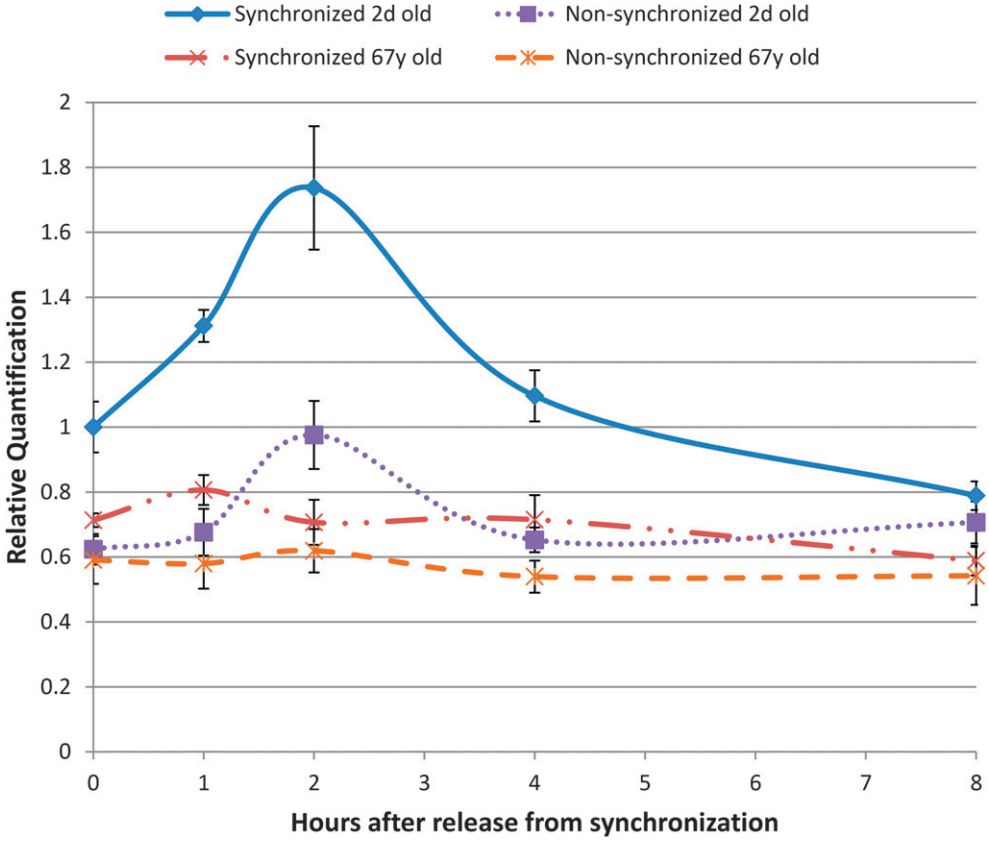

Figure 2. Cell synchronization was induced by nutrient deprivation followed by nutrient repletion, which induced a temporal rhythm that showed an increase in autophagy by 77.9% (±10.9% SEM) in young fibroblasts, whereas this effect was absent in aged fibroblasts. LC3B expression was determined by RT-PCR and normalized to GAPDH housekeeping genes (n = 3).

RESULTS

Normal human dermal skin fibroblasts derived from either 2-day-old or 67-year-old donors were probed with antibodies directed against LC3B antigens for determining the level of autophagy. Immunocytofluorescence revealed a marked decrease in autophagy in the mature fibroblasts as compared to the young fibroblasts [23.8% ± 1.9% standard error of mean (SEM)] and these data are shown in Figures 1A and B. When fibroblasts were synchronized by nutrient deprivation and then repleted with full media, significant increases over time (77.9% ± 10.9% SEM) in LC3B transcripts as determined by RT-PCR were measured after 2 h in the young fibroblasts, whereas in the aged fibroblasts this effect was absent (Figure 2). Interestingly, we also observed this relationship in the unsynchronized cells albeit to a much lesser degree.

Under both conditions, we found a distinct biphasic temporal pattern, which correlated to the night in Zeitgeber time. As a control to ensure that our model system was responsive to changes in circadian patterns, the clock gene transcript for *per2* was also evaluated by RT-PCR. The results from this experiment showed a temporal pattern that correlated with the evening onset of this clock gene (8) and are shown in Figure 3. Thus, an increase in autophagy in young cells occurs in the evening that is not evident in mature cells. These data were also supported by cellular fluorescent images captured over time under the same conditions for LC3B as shown in Figure 4 and clearly demonstrate higher levels of autophagy for young fibroblasts at night.

Since it had been previously reported that oxidative stress is a potent activator of autophagy (9) and that sunlight is a source of this type of stress, we then exposed fibroblasts to 10 J/cm^2 UVA and determined autophagy over time as before. In young cells under synchronization conditions, LC3B expression levels after irradiation were found to be similar to their endogenous levels. However, in mature cells, LC3B expression could be induced by 27.2% (±5.3% SEM) and the phase of the induction of autophagy was shifted earlier by 1 h. As shown in Figure 4, these data demonstrate a potential for reactivation of autophagic mechanisms.

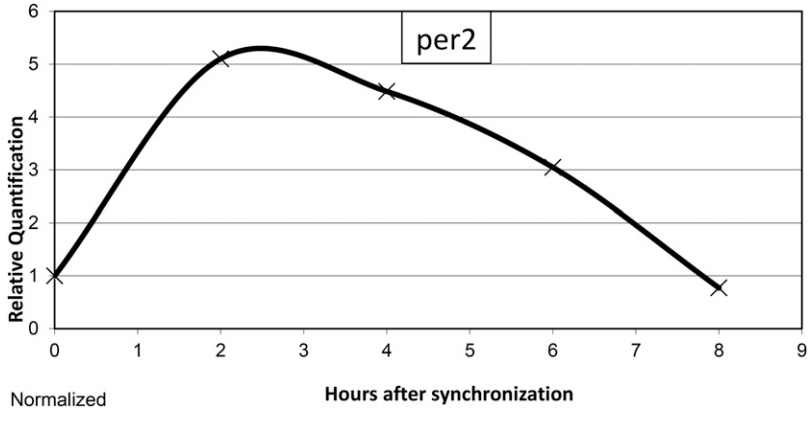

Figure 3. *Per2* expression that increases in the evening was assayed by RT-PCR over time in parallel to the synchronization experiments in young fibroblasts to confirm that evening Zeitgeber time in our experiments peaked at approximately 2 h after nutrient repletion (n = 3). Expression normalized to GAPDH housekeeping genes.

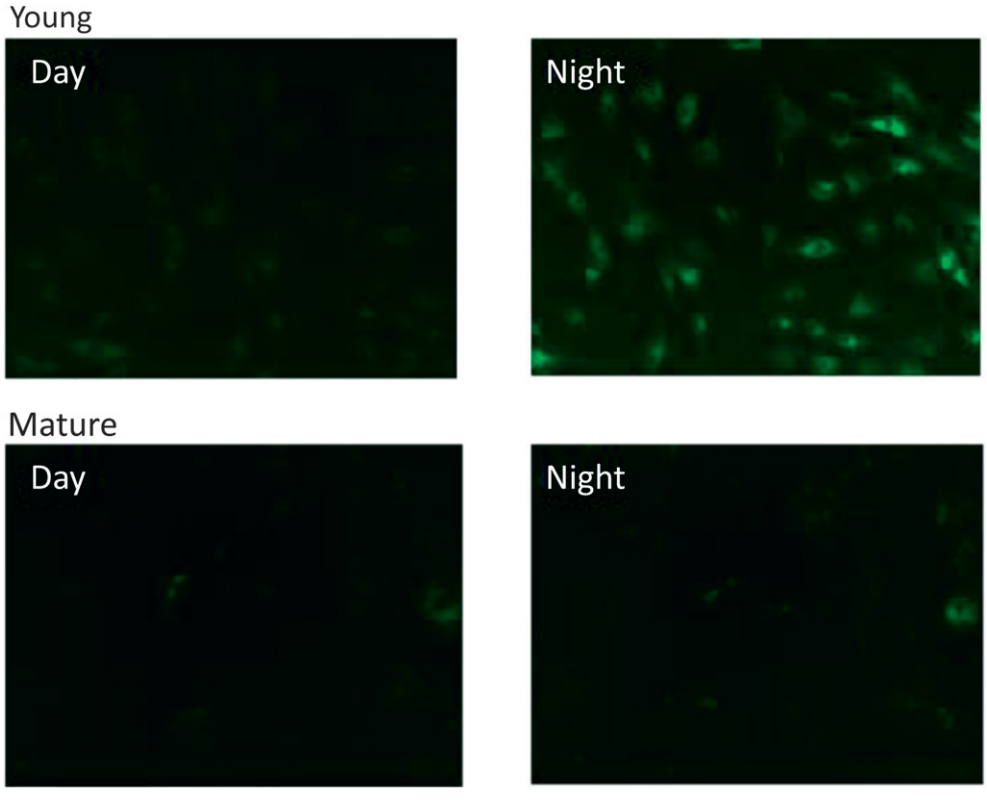

Figure 4. Immunocytofluorescence over time of young and aged fibroblasts after release from synchronization show an increase in LC3B/autophagy in young cells associated with night but not in mature cells (magnification = 20×).

DISCUSSION

It is now more than 50 years since autophagy was first characterized as a basic cellular process by de Duve (10). During this time, great strides have also been made in aging research (11), as well as in understanding the importance of circadian rhythm in maintaining cellular health (12), as well as in human skin cells (13). In this report, we bring these disciplines together to demonstrate that autophagy follows a temporal pattern in young, normal human dermal fibroblasts, which supports other work in this field as reviewed by Sachdeva and Thompson (6). We further show a significant reduction in autophagy as a function of age in mature fibroblasts and correlate this decrease to a concomitant loss of autophagosomal rhythm as reflected by reduced levels of LC3B over time. Moreover, others have shown a connection to autophagy and circadian rhythm. Ma *et al.* (14) found that autophagy was disrupted in liver cells that lacked a functional biological clock and Weibking *et al.* (15) observed decreased autophagic factors in *per1*-/- knockout mice.

In aging cells, due to their dissynchronization with circadian rhythm, much damage will accumulate over time accelerating the aging process. Since disruption of circadian cycles can lead to various pathologies, as observed by a rise in cancer rates in shift workers (16), understanding and restoring these cycles will lead to increased health benefits. In skin,

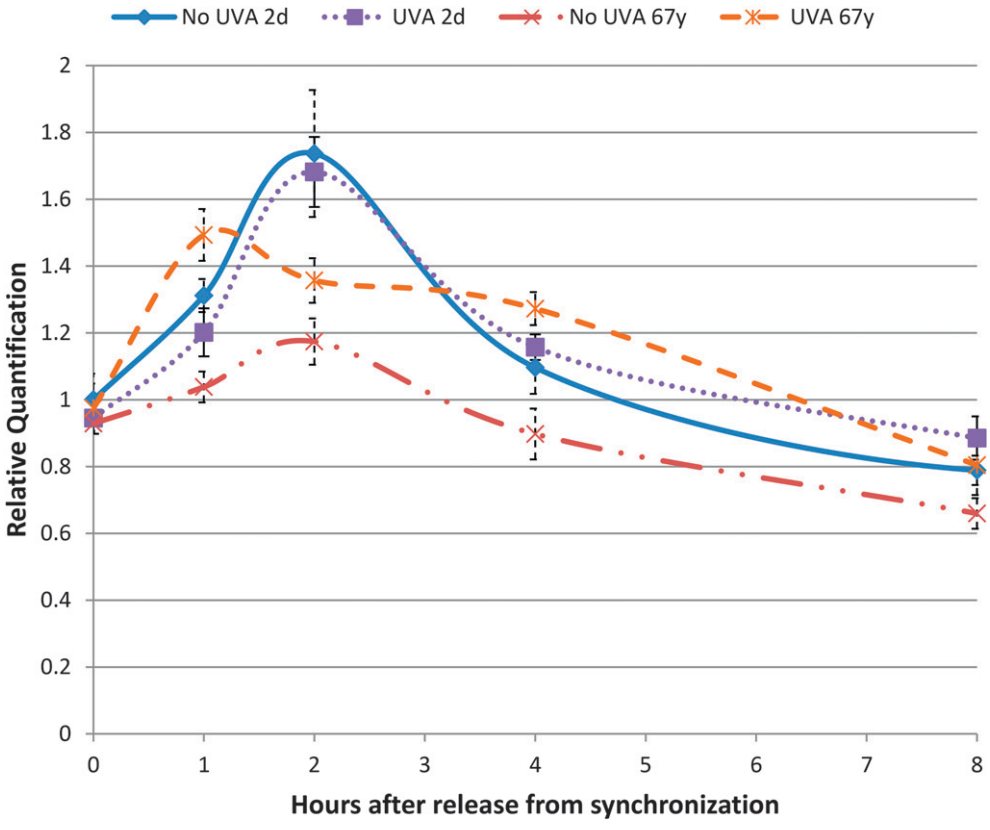

Figure 5. UVA irradiation of fibroblasts did not significantly affect LC3B/autophagy levels in young cells. However, UVA significantly increased LC3B/autophagy in mature cells by 27.2% (±5.3% SEM) and phase shifted its peak expression to 1 h.

improvement in autophagy function may also lead to amelioration in the visible signs of cutaneous aging. Interestingly, after UV irradiation of cells, we also observed a slight reinduction of the autophagosomal response in those cells derived from mature donors. Thus, our data indicate the possibility of restoring autophagosomal function in aged skin. Since a phase shift in the timing of autophagy was also observed, resynchronization as a treatment modality should also be investigated.

Future research should include investigation into the downstream effects of UV exposure in skin such as the generation of reactive oxygen species and how they may contribute to the activation of intracellular damage removal. In addition, oxidation-sensitive signaling cascades, such as mitogen-activated protein kinases (MAPK), which can lead to genetic activation of antioxidant response elements, may also be a productive area for future studies and the utilization of fibroblasts from more donors should strengthen our conclusions. In conclusion, we report for the first time that autophagy levels peak at night in young fibroblasts, whereas in aged human skin fibroblasts, there is a reduction in autophagy. We further show the effect that environmental stress has on the expression of autophagic processes, and demonstrate how autophagy may be potentiated under these conditions, which emphasize its importance in maintaining healthy skin.

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