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Mutation in keratin 18 induces mitochondrial fragmentation in liver-derived epithelial cells

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Abstract

Microtubules (MTs) and microfilaments (MFs) are known to modulate mitochondrial morphology, distribution and function. However, little is known evidence about the role of intermediate filaments (IFs) in modulating mitochondria except desmin. To investigate whether or not the IFs regulate mitochondrial morphology, distribution, and function, we manipulated the IFs of cultured epithelial cells to express a mutant keratin 18 (K18). In contrast to the filamentous expression of wild K18, mutant K18 induced aggregation of K8/18, showing no fine IF network in the cells. In mutant K18-transfected cells, the mitochondria were fragmented into small spheroids, although they were observed as mitochondrial fibers in un-transfected or wild K18-transfected cells. Fluorescence recovery after photobleaching of fluorescence-labeled mitochondria was markedly less in the mutant K18-transfected cells, although a significant recovery was confirmed in wild K18-transfected cells. These findings suggest that the IFs are important for the maintenance of normal mitochondrial structures.

Keywords: Cytoskeleton; Hepatocyte; Intermediate filament; Keratin; Microfilament; Microtubules; Mitochondria

The mitochondria are complex organelles with a double membrane, their own genome, and a separate protein synthetic machinery. They perform a variety of important functions including regulations of the tricarboxylic acid cycle and ATP production, Ca homeostasis, oxidative phosphorylation and intracellular signaling including apoptosis [1–4]. They exist as a network with a tubule-like morphology and maintain their shape by movement, fission and fusion. Recently proteins that control mitochondrial dynamics have been identified. They include optic atrophy 1 (OPA1)[1] and mitofusions involved in fusions, and dynamin-related protein 1 (Drp 1) involved in fission [2,4–8].

The cytoskeleton of mammalian cells plays a pivotal role in cellular functions. The cytoskeleton consists of three major protein families, microtubules (MTs), microfilaments (MFs) and intermediate filaments (IFs), and their associated proteins. Organelle transport is widely considered to be a cytoskeleton dependent process [9–13]. Studies

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[1] *Abbreviation used:* CB, cytochalasin B; DMSO, dimethyl sulfoxide; Drp1, dynamin-related protein 1; FRAP, fluorescence recovery; GFP, green fluorescent protein; IF, intermediate filament; K, keratin; MF, microfilament; MT, microtubule; OPA1, optic atrophy 1; WT, wild type.

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in a wide variety of cell types indicate that mitochondria co-localized with MFs, MTs and IFs, and mitochondria move through the cytoplasm using both MT-based and MF-based motor activities [4,9,10,13,14]. However, the relation between the mitochondria and the IFs has been unclear except in the case of desmin [15].

IFs consist of more than 70 unique gene products that make up a highly dynamic family of cytoplasmic and nuclear disease-associated cytoskeletal proteins. The roles of IFs in protection from mechanical and non-mechanical stresses distinguishes them from other cytoskeletal elements. This cytoprotective function is reflected by numerous human disease phenotypes that are associated with mutations of genes encoding IFs [16]. The components of IFs of epithelial cells are keratins [12,16–18]. Keratin mutations are associated with several skin, oral, esophageal, ocular, hair, and liver diseases that reflect the tissue-specific expression of the particular keratins [16]. Emerging evidence indicates that keratins protect cells from nonmechanical injury via mechanisms that include keratin regulation of cell signaling cascades, regulation of susceptibility to apoptosis, and modulation of protein targeting to subcellular compartments [19,20]. Adult hepatocytes are unique, because they express keratin 8/18 (K8/18) exclusively [12,17,18]. Some K8/K18 mutations predispose to a risk for liver disease progression [17,21–24].

In skeletal and cardiac muscle, the surface of desmin IFs bridging the interfibrillar space between neighboring Z discs are skimmed with mitochondria [25]. Association of desmin bundles with mitochondria in smooth muscles has been also reported [25–27]. They play key role in determining different mitochondrial morphologies and distributions, and are also required for mitochondrial movements. Neurofilaments are associated with mitochondria including transport of mitochondria [28] and this association depends on mitochondrial membrane potential [29]. Mutation of K5 affected the distribution of the mitochondria [30]. Therefore, it is conceivable that K8/18 is associated with mitochondria in hepatocytes.

Morphological changes of mitochondria were reported in several diseases. Megamitochondria have been observed in alcoholic liver disease and various other liver diseases [27,31–33]. Mitochondria undergo extensive fragmentation during apoptosis [8]. Furthermore, mitochondria need to be localized at particular sites for providing energy supply and for participating in intracellular signaling [3,4]. Thus, the shape and distribution of the mitochondria are apparently associated with their functions [3,8].

Highly conserved K18 arginine 89 play an important role in keratin organization [34] and expression of the mutant protein in cultured cells induces loss of fine IF network [35]. In this study, to clarify the role of IFs in mitochondrial morphology and movements, we manipulated the IF network by expressing a mutant K18 arg89cys (K18 R89C) tagged with green fluorescent protein (GFP) in liver-derived epithelial cells.

Materials and methods

Cell lines and cell culture. Huh7 and OUMS29 cells were cultured in DMEM (Sigma, St. Louis, MO) supplemented with 10% FCS (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and antibodies. At 24 h after the plating, cDNAs were transfected into the cell lines using Effecten Transfection Reagent according to the manufacturer's recommendations (Qiagen, Hilden, Germany).

Preparation of plasmids. GFP-wild type (WT) K18 and GFP-K18 R89 C were constructed as previously described [35,36]. pDsRed2-Mito was purchased from BD Biosciences Clonetech (Palo Alto, CA, USA).

Antibodies and reagents. The following antibodies were used: mouse monoclonal anti-actin and anti-tubulin antibodies (Sigma); mouse monoclonal anti-K type I and II antibodies (Progen Biotechnik GMBH, Heidelberg, Germany); tetramethylrhodamine isothiocyanate-conjugated rabbit anti-mouse immunoglobulins (Dako Japan, Tokyo, Japan).

The followings were purchased from Sigma, and used at the specified dose: nocodazole (10 μM) and cytochalasin B (CB) (50 μM). Stock solutions were prepared as follows and stored at −20 °C: nocodazole, 300 mM in dimethyl sulfoxide (DMSO); CB, 50 mM in DMSO.

Immunofluorescence microscopy. At 48 h after the transfection, cells were fixed in 3% paraformaldehyde in phosphate buffer saline for 30 min, and permeabilized in 0.2% Triton X-100 in phosphate buffer saline. After the blocking, cells were incubated with the primary antibodies for 1 h and the secondary antibodies for 1 h. For the examination of inhibition of MTs or MFs on mitochondrial morphology, cells were treated with nocodazole for 6 h or CB for 3 h, respectively, before the fixation.

A confocal laser scanning microscope (Fluoview FV 300; Olympus, Tokyo, Japan) equipped with an Argon/Krypton laser capable of dual excitation and detection was used to visualize the distribution of GFP, DsRed and immunostainings.

Fluorescence recovery after photobleaching. To monitor the mobility of the mitochondria, we applied the fluorescence recovery after photobleaching (FRAP) method using cells co-expressing GFP-WT K18 or K18 R89C with DsRed-Mit. Cellular areas typically 25 μm^2 were bleached by briefly digitally zooming into the region of interest with enhanced laser intensity. Fluorescence intensity of DsRed-Mit were measured before, immediately after, and 2 h after the bleaching in the bleached and control areas, and their ratios were calculated, because the fluoresce intensity of the cells as a whole was decreased during the process of scanning and bleaching.

Morphometric analysis. The distribution of the mitochondria was classified into 2 types: scattered pattern when they were distributed throughout the cell as normal mitochondria and accumulated pattern when they were accumulated near the nucleus.

The morphology of the mitochondria was classified into 2 types: filamentous pattern, when they existed as a network with a tubule-like morphology (or mitochondrial fiber) and fragmented pattern when they existed as small spheroids. We examined 100 cells in each group from 4 independent experiments.

Statistical analysis. The results are expressed as mean ± SD Data were analyzed with the Student's *t*-tests and Fisher's PLSD test. A p-value <0.05 was considered as significant.

Results

Co-expression of GFP-WT K18 and DsRed-Mito in Huh7 cell

The expression of GFP-WT K18 revealed a fine intracellular network and it was incorporated into the endogenous IFs. When GFP-WT K18-transfected cells were stained with anti-pan-Keratin antibodies, GFP signals and immunoreactive signals were completely coincident (Fig. 1a–c). DsRed-Mito was distributed throughout the cytoplasm

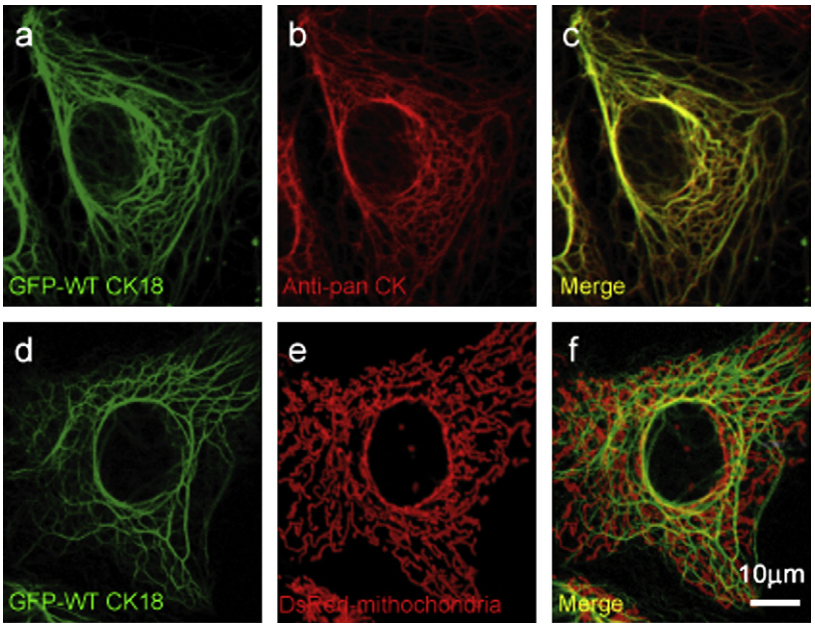

Fig. 1. Confocal laser scanning microscopic images of GFP-WT K18-transfected- (a–c), and GFP-WT K18 and DsRed Mito co-transfected-Huh7 cells (d–f). IFs were labeled with anti-pan-Ks (a–c) antibodies. GFP signals and TRITC signals were completely colocalized and showed a filamentous network of IFs (a–c). The co-expression of GFP-WT K18 and DsRed-Mito had no interaction between each expressed organelles (d–f). Bar, 10 μm. Green (a, c, d and f) GFP-WT K18; red (b and c) anti-pan-Ks, (e and f) DsRed-Mito.

and they were observed as mitochondrial fibers in the transfected cells (Fig. 1d–f).

The effect of MTs or MFs disruption on the morphology and distribution of mitochondria in Huh7 cells

We examined the morphology and distribution of mitochondria after the treatments with nocodazole or CB, which inhibits MTs or MFs, respectively, in GFP-WT K18 and DsRed-Mito co-transfected cells. The present dose of nocodazole and CB induced disappearance of MT and MF networks as examined by immunofluorescence (data not shown). Both nocodazole and CB caused fragmentation of mitochondrial tubule-like morphology, but did not induce any apparent change in the distributions of mitochondria or the filamentous network of IFs (Fig. 2a–f, Tables 1 and 2). Fragmented mitochondria were distributed throughout the cytoplasm (Fig. 2a–f). DMSO alone did not affect the mitochondrial distribution (data not shown).

Co-expression of GFP-K18 R89C and DsRed-Mito

We examined the effect of expression of the mutant K18 (K18 R89C) on the distribution and morphology of the mitochondria in Huh7 and OUMS29 (data not shown) cells. The co-transfection of GFP-K18 R89C and DsRed-Mito resulted in perinuclear IF aggregation and fragmentation of mitochondria (Fig. 2g–i, Table 1), however, it did not affect the distribution of mitochondria in either cell type. They were observed throughout the cytoplasm (Fig. 2g–i, Table 2).

FRAP of DsRed-mitochondria in cytoskeleton-inhibited cells

To identify the roles of cytoskeletons in mitochondrial transport, we performed FRAP experiments. The DsRed fluorescence was bleached in a sub-cellular region using brief high intensity illumination in GFP-K18 and DsRed-Mito co-expressing Huh7 cells, and we examined the mitochondrial red signals before, immediately after and at 2 h after bleaching. Intensity of DsRed-Mito recovered significantly 2 h after bleaching in GFP-K18 WT-transfected cells (Fig. 3a–c). However, the fluorescent intensity did not recover in cells pre-treated with nocodazole (Fig. 3d–f) or CB (Fig. 3g–i). DsRed-Mito intensity did not recover at 2 h after bleaching in DsRed-Mito and GFP-K18 R89C co-transfected cells (Fig. 3j–l). Measurement of fluorescent intensity demonstrated that the intensity decreased significantly after the bleaching and then recovered after 2 h in the control but not in the cytoskeleton inhibited groups (Fig. 4).

Discussion

The association of mitochondria with the cytoskeleton has been noted for many years, and numerous studies have suggested that the cytoskeletons are involved in the movement and localization of mitochondria as well as other organelles and cellular components [4,9–11,13,25]. However, in contrast to MTs and MFs, it is not clear whether

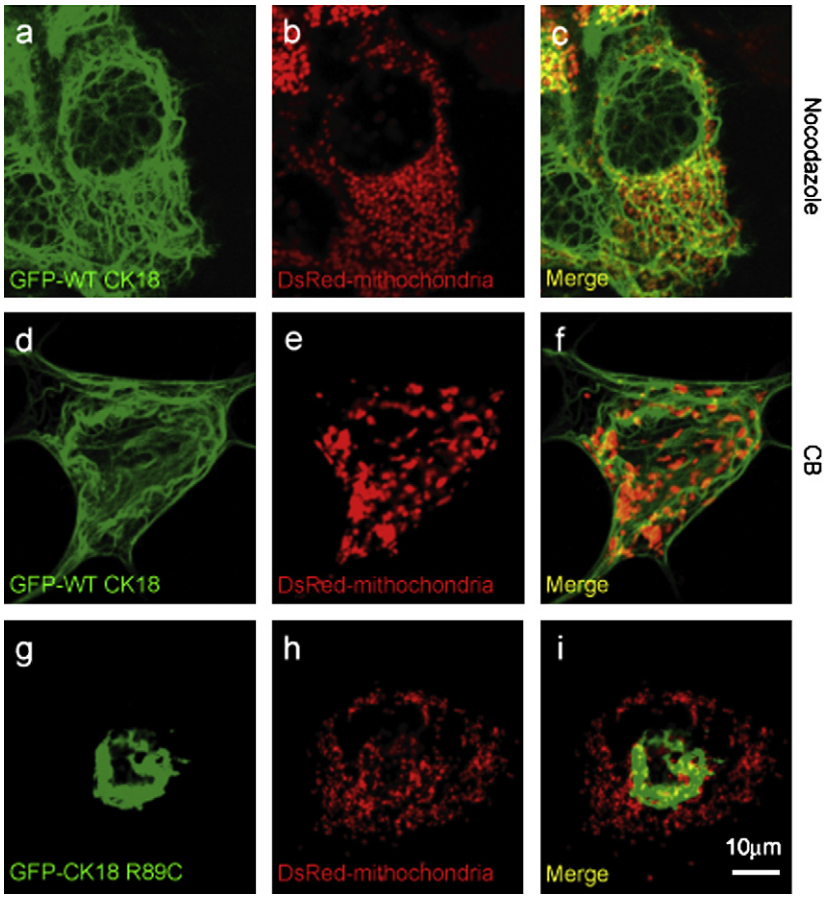

Fig. 2. Confocal laser scanning microscopic images of GFP-WT K18 and DsRed-Mito co-transfected Huh7 cells (a–f). Cells were treated with nocodazole for 6 h (a–c) or CB for 3 h (d–f). The treatments caused fragmentation of the mitochondrial network, but, did not change the distribution of mitochondria. Confocal laser scanning microscopic images of GFP-K18 R89C and DsRed-Mito co-transfected Huh7 cells (g–i). The expression of GFP-K18 R89C resulted in IF aggregates (g) and caused mitochondrial fragmentation, but did not alter mitochondrial distribution (h). Green (a, c, d and f) GFP-WT CK18; GFP-K18 R89C; red (b, c, e, f, h and i) DsRed-Mito.

Table 1
The relative ratio of mitochondrial fragmentation in Huh7 cells

Transfected cDNA	Treated agents		
	No treatment	Cytokalasin B	Nocodazole
GFP-WT CK18 (%: mean ± SD)	19.8 ± 6.62	74.9 ± 8.13*	56.5 ± 7.945*
GFP-CK18 R89C (%: mean ± SD)	79.2 ± 23.4*		

The relative ratio of mitochondrial fragmentation is expressed as the percentage of the total number of cells counted in each group. Data were from ten independent experiments.

* $p < 0.05$ compared with GFP-WT CK18-transfected cells.

Table 2
The relative ratio of mitochondrial accumulation in Huh7 cells

Transfected cDNA	Treated agents		
	No treatment	Cytokalasin B	Nocodazole
GFP-WT CK18 (%: mean ± SD)	10.5 ± 3.33	25.4 ± 28.3	20.8 ± 16.6
GFP-CK18 R89C (%: mean ± SD)	17.1 ± 6.71		

The relative ratio of mitochondrial accumulation is expressed as the percentage of the total number of cells counted in each group. Data were from ten independent experiments.

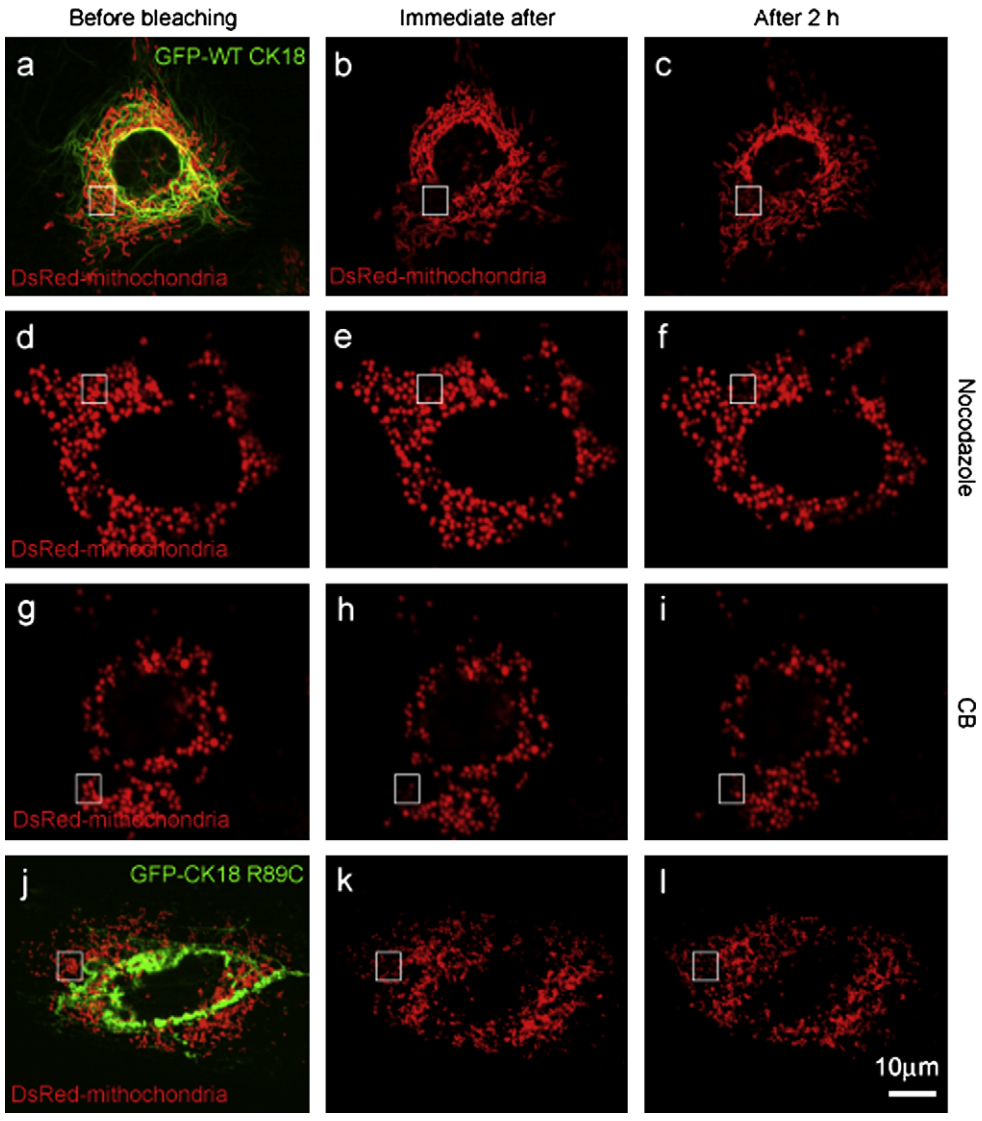

Fig. 3. Confocal laser scanning microscopic images of GFP-WT K18 (a–i) or GFP-K18R89C (j–l) and DsRed-Mito co-trasfected-Huh7 cell. Cells were treated with nocodazole (d–f) or CB (g–i). FRAP experiments were conducted in each group, and the time course of the signal from DsRed-Mito were chased before (a, d, g and j), immediately after (b, e, h and k) and at 2 h after (c, f, i and l) the bleaching. The intensity of bleached area of mitochondria recovered in GFP-WT K18-transfected cells, however, it did not recover in nocodazole or CB treated or GFP-K18 R89C-transfected cells. Red (a–l) DsRed-mitochondria; green (j–l) GFP-K18 R89C.

IFs are required for mitochondrial distribution because of the lack of a specific inhibitor.

We have reported that the expression of GFP-K18 R89C resulted in loss of the filament network composed of K8/18 and induced redistribution of various intracellular proteins in liver-derived epithelial cells [35,37]. Therefore, we extended these findings by examining mitochondrial morphology and distribution in GFP-K18 R89C-transfected cells. We found that inhibition of MTs, MFs or IFs caused fragmentation of the mitochondrial network. To investigate the relation of the mitochondrial transportation and cytoskeletons in more detail, we conducted FRAP experiments. The photobleached fluorescent intensity of DsRed-Mito showed less fluorescence recovery in all cytoskeleton-inhibited cells.

MF and MT systems cooperate for intracellular organelles. The long-range transport of cellular components in animal cells is based on MT networks, whereas the actin network appears to be critical for short-range transport [4,10,11,13]. The distribution of fragmentated mitochondia was not affected by the treatment of MTs or MF. Mitochondria are related to both the kinesin and dynein families, and MFs are important for short distance movement of mitochondria [4,13]. Mitochondria undergo both anterograde and retrograde movement along microtubule and actin track [38]. Therefore nocodazole may inhibit both anterograde and retrograde movement and CB may inhibit bidirectional short distance movement. However, both are important for the mitochondrial transport and fusion process. The morphology of the mitochondria was similarly

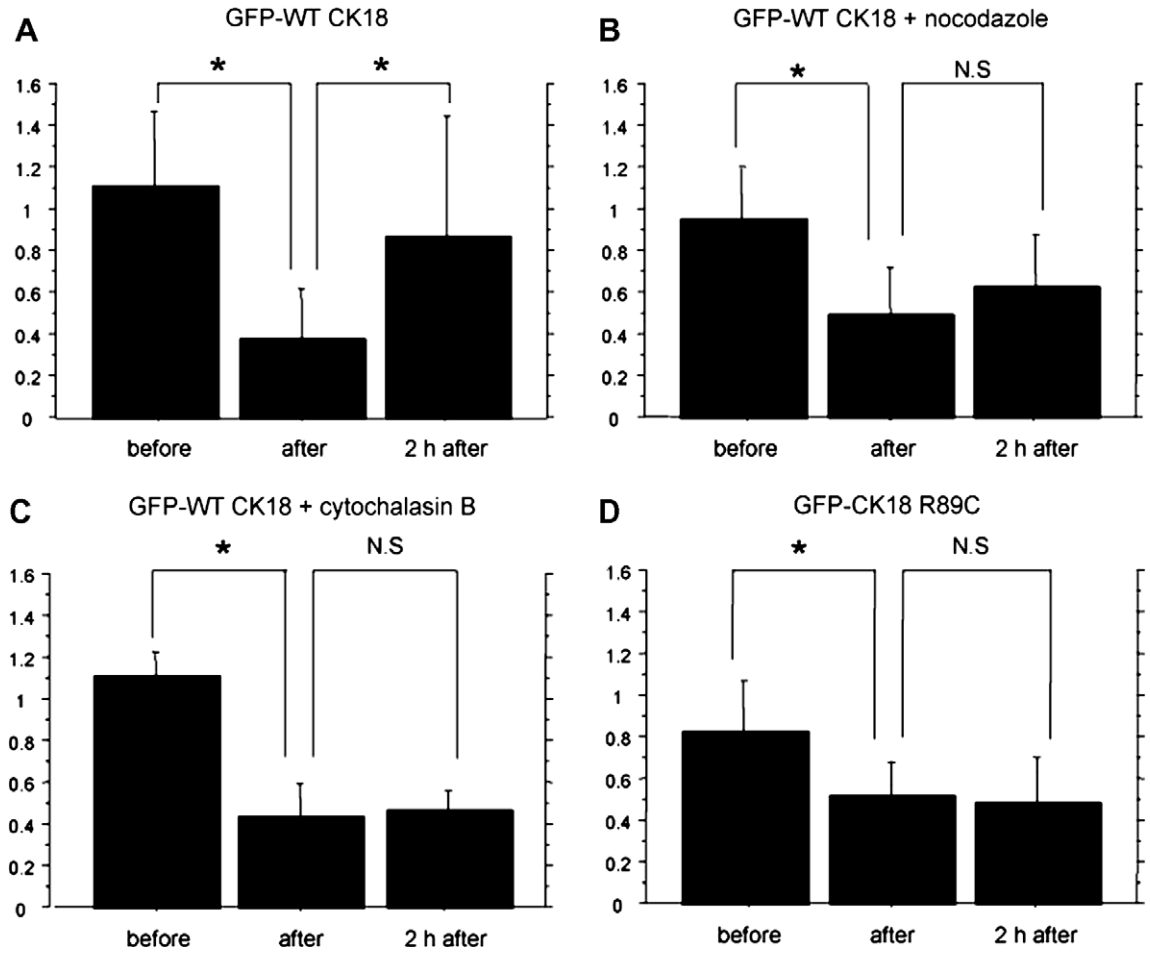

Fig. 4. Fluorescence recovery after photobleaching in cytoskeleton inhibited Huh7 cells. FRAP experiments were perfomed in 30 cells in each group. The *Y* axis indicates the relative intensity of photobleached area of DsRed-Mito compared to non-photobleached area. Mitochondrial intensity recovered significantly in the control group, however, it did not recover in cytoskeleton-inhibited groups. (A) GFP-WT K18 transfected Huh7 cell, (B) GFP-WT K18 transfected Huh7 cell treated with nocodazole, (C) GFP-WT K18 transfected Huh7 cell treated with CB, (D) GFP-K18 R89C transfected Huh7 cell. $^{*}p < 0.05$.

changed in mutant K18-expressing cells. Our previous study demonstrated that the expression of this mutant did not affect the distribution of MTs and MF [35]. Therefore, the fine IF network apparently plays some role in the maintenance of normal mitochondrial shapes.

Mitochondrial dysfunction can contribute to cytotoxicity and oxidative stress and has been implicated in some neurodegenerative diseases [39]. Morphological changes of the mitochondria and inclusion body were reported in several liver diseases [32,33,40,41]. The morphological changes of mitochondria reflect cellular injury that can be identified by the impairment of mitochondrial electron transport chain or alteration in oxidative process [42,43]. Recently, Campbel et al. reported that decreased actin dynamics cause depolarization of mitochondrial membrane and an increase in production of reactive oxygen species, resulting in cell death, and that increasing actin dynamics can increase lifespan [44]. Several studies described the relationship between the morphology and function of mitochondria [4,8]. Mitochondria in desmin-null muscle demonstrated abnormal accumulation, proliferation and swelling, and mitochondrial respiration *in situ* was significantly reduced [25,27]. However, the main morphological change of mitochondria in the present study was fragmentation from normal tubule like morphology (mitochondrial fiber). Mitochondrial fragmentation is associated with apoptosis and the mitochondrial fission/fusion process is important for apoptosis as upstream process of caspase activation [8]. The mitochondrial fission *per se* does not induce apoptosis, but is integral step of apoptosis [8]. Therefore, this situation may be a predisposing condition for apoptosis after further stimulation or stress. Mutation in K8/K18 is not the direct cause of liver disease but predisposes for the subsequent development of cirrhosis and liver disease progression [17,21,23,24]. It is possible that the change in mitochondrial shape affects the disease progression from various stimuli and may in part explain the relation between mutation of K8/18 and liver disease progression.

In conclusion, we investigated the effect of inhibition of cytoskeletons on mitochondrial morphology, by the expression of mutant K18 and treatments with MT or MF inhibitors. We demonstrated that inhibition of all

three cytoskeleton structures caused mitochondrial fragmentation, and that fragmented portions in the photobleached mitochondria showed less fluorescence recovery in these conditions. These findings suggested that the cytoskeletons, including IF, play important role in maintenance of mitochondrial morphology, especially the fusion and transport process, and this function must be important for cell integrity.

Acknowledgments

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